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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 38/00, 31/70, C07K 5/00, 7/00, 16/00, 17/00, C12P 21/06, 21/08, C07H 21/04, A01N 37/18, 43/04, G01N 33/48, 33/53, 33/574		A3	(11) International Publication Number: WO 00/02897 (43) International Publication Date: 20 January 2000 (20.01.00)
(21) International Application Number: PCT/US99/15817 (22) International Filing Date: 13 July 1999 (13.07.99)		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 60/092,513 13 July 1998 (13.07.98) 60/104,834 19 October 1998 (19.10.98)		US US	Published <i>With international search report.</i> <i>With amended claims.</i>
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(54) Title: DELTA CLEAVAGE PRODUCTS AND METHODS BASED THEREON

(57) Abstract

The present invention is directed to a Delta cleavage peptide and fragments, derivative and analogs thereof, and its encoding nucleic acids. The present invention is also directed to a soluble Delta peptide and fragments, derivative and analogs thereof, and its encoding nucleic acids. The present invention is also directed to protein complexes of Delta and Kuz. The present invention is also directed to methods for detecting or measuring Delta activation by observing or measuring Delta cleavage products that are indicative of Delta activation. The present invention is also directed to methods for detecting or measuring Kuz activation by observing or measuring Delta cleavage products that are indicative of Kuz activation. The present invention is also directed to methods for detecting a molecule that modulates Delta activation or Kuz function by observing or measuring a change in the amount of or pattern of Delta cleavage products. The present invention is based, at least in part, on the discovery that Delta in its active form, i.e., the form that mediates signal transduction and that binds Notch, is a soluble fragment consisting of the extracellular domain.

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DELTA CLEAVAGE PRODUCTS AND METHODS BASED THEREON

The application claims priority benefits of U.S. Provisional Application Serial No. 60/104,834, filed October 5 19, 1998 and U.S. Provisional Application Serial No. 60/092,513 filed July 13, 1998, each of which is incorporated by reference herein in its entirety.

1. FIELD OF THE INVENTION

10 The present invention is directed to a peptide, and its encoding nucleic acids, of the toporythmic protein Delta that contains a sequence which is cleaved by the metalloprotease-disintegrin Kuzbanian (Kuz) ("Delta cleavage peptide"), as well as derivatives and analogs thereof. The present invention is also directed to an extracellular 15 soluble peptide, and its encoding nucleic acids, of the toporythmic protein Delta ("soluble Delta peptide" or "Dl^{EC}"), as well as derivatives and analogs thereof. Production of the Delta cleavage peptide or Dl^{EC}, and derivatives, and antibodies thereto are also provided. The present invention 20 is also directed to methods for detecting or measuring Delta activation by observing or measuring Delta cleavage products that are indicative of Delta activation. The present invention is also directed to methods for detecting a molecule that modulates Delta activation by observing or 25 measuring a change in the amount or pattern of Delta cleavage products. The present invention is further directed to methods for detecting or measuring Kuz function by observing or measuring Delta cleavage products that are indicative of Kuz function. The present invention is also directed to methods for detecting a molecule that modulates Kuz function 30 by observing or measuring a change in the amount or pattern of Delta cleavage products. The present invention is also directed to certain protein complexes of Delta and Kuz and of

D₁^{EC} and Notch, and methods for their use in screening, diagnosis and therapy.

2. BACKGROUND OF THE INVENTION

5 Genetic and molecular studies have led to the identification of a group of genes which define distinct elements of the Notch signaling pathway. While the identification of these various elements has come exclusively from *Drosophila* using genetic tools as the initial guide, 10 subsequent analyses have lead to the identification of homologous proteins in vertebrate species including humans. Figure 1 depicts the molecular relationships between the known Notch pathway elements as well as their subcellular localization (Artavanis-Tsakonas et al., 1995, *Science* 268:225-232).

15 The *Drosophila* Notch gene encodes an ~300 kD transmembrane protein that acts as a receptor in a cell-cell signaling mechanism controlling cell fate decisions throughout development (reviewed, e.g., in Artavanis-Tsakonas et al., 1995, *Science* 268:225-232). Closely related homologs 20 of *Drosophila* Notch have been isolated from a number of vertebrate species, including humans, with multiple paralogs representing the single *Drosophila* gene in vertebrate genomes. The isolation of cDNA clones encoding the C-terminus of a human Notch paralog, originally termed hN, has 25 been reported (Stifani et al., 1992, *Nature Genetics* 2:119-127). The encoded protein is designated human Notch2 because of its close relationship to the Notch2 proteins found in other species (Weinmaster et al., 1992, *Development* 116:931-941). The hallmark Notch2 structures are common to all the 30 Notch-related proteins, including, in the extracellular domain, a stretch of 34 to 36 tandem Epidermal Growth Factor-like (EGF) repeats and three Lin-12/Notch repeats (LN repeats), and, in the intracellular domain, 6 Ankyrin repeats.

and a PEST-containing region. Like *Drosophila Notch* and the related *C. elegans* genes *lin-12* and *glp-1* (Sternberg, 1993, Current Biology 3:763-765; Greenwald, 1994, Current Opinion in Genetics and Development 4:556-562), the vertebrate Notch 5 homologs play a role in a variety of developmental processes by controlling cell fate decisions (reviewed, e.g., in Blaumueller and Artavanis-Tsakonas, 1997, Persp. on Dev. Neurobiol. 4:325-343). (For further human Notch sequences, see International Publication WO 92/19734.)

10 The extracellular domain of Notch carries 36 Epidermal Growth Factor-like (EGF) repeats, two of which (repeats 11 and 12) have been implicated in interactions with the Notch ligands Serrate and Delta. Delta and Serrate are membrane bound ligands with EGF homologous extracellular domains, which interact physically with Notch on adjacent 15 cells to trigger signaling.

Functional analyses involving the expression of truncated forms of the Notch receptor have indicated that receptor activation depends on the six *cdc10/ankyrin* repeats in the intracellular domain. Deltex and Suppressor of 20 Hairless, whose over-expression results in an apparent activation of the pathway, associate with those repeats.

Deltex is a cytoplasmic protein which contains a ring zinc finger. Suppressor of Hairless on the other hand, is the *Drosophila* homologue of CBF1, a mammalian DNA binding 25 protein involved in the Epstein-Barr virus-induced immortalization of B cells. It has been demonstrated that, at least in cultured cells, Suppressor of Hairless associates with the *cdc10/ankyrin* repeats in the cytoplasm and translocates into the nucleus upon the interaction of the Notch receptor with its ligand Delta on adjacent cells 30 (Fortini and Artavanis, 1994, Cell 79:273-282). The association of Hairless, a novel nuclear protein, with Suppressor of Hairless has been documented using the yeast

two hybrid system; therefore, it is believed that the involvement of Suppressor of Hairless in transcription is modulated by Hairless (Brou et al., 1994, *Genes Dev.* 8:2491; Knust et al. 1992, *Genetics* 129:803).

5 Finally, it is known that Notch signaling results in the activation of at least certain basic helix-loop-helix (bHLH) genes within the Enhancer of Split complex (Delidakis et al ., 1991, *Genetics* 129:803). Mastermind encodes a novel ubiquitous nuclear protein whose relationship to Notch signaling remains unclear but is involved in the Notch 10 pathway as shown by genetic analysis (Smoller et al., 1990, *Genes Dev.* 4:1688).

15 The generality of the Notch pathway manifests itself at different levels. At the genetic level, many mutations exist which affect the development of a very broad 20 spectrum of cell types in *Drosophila*. Knockout mutations in mice are embryonic lethals consistent with a fundamental role for Notch function (Swiatek et al., 1994, *Genes Dev.* 8:707). Mutations in the Notch pathway in the hematopoietic system in humans are associated with lymphoblastic leukemia (Ellison et 25 al., 1991, *Cell* 66:649-661). Finally the expression of mutant forms of Notch in developing *Xenopus* embryos interferes profoundly with normal development (Coffman et al., 1993, *Cell* 73:659). Increased level of Notch expression is found in some malignant tissue in humans (International Publication WO 94/07474).

25 The expression patterns of Notch in the *Drosophila* embryo are complex and dynamic. The Notch protein is broadly expressed in the early embryo, and subsequently becomes restricted to uncommitted or proliferative groups of cells as development proceeds. In the adult, expression persists in 30 the regenerating tissues of the ovaries and testes (reviewed in Fortini et al., 1993, *Cell* 75:1245-1247; Jan et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:8305-8307; Sternberg, 1993,

Curr. Biol. 3:763-765; Greenwald, 1994, Curr. Opin. Genet. Dev. 4:556-562; Artavanis-Tsakonas et al., 1995, Science 268:225-232). Studies of the expression of Notch1, one of three known vertebrate homologs of Notch, in zebrafish and 5 *Xenopus*, have shown that the general patterns are similar; with Notch expression associated in general with non-terminally differentiated, proliferative cell populations. Tissues with high expression levels include the developing brain, eye and neural tube (Coffman et al., 1990, Science 10 249:1438-1441; Bierkamp et al., 1993, Mech. Dev. 43:87-100). While studies in mammals have shown the expression of the corresponding Notch homologues to begin later in development, the proteins are expressed in dynamic patterns in tissues undergoing cell fate determination or rapid proliferation (Weinmaster et al., 1991, Development 113:199-205; Reaume et 15 al., 1992, Dev. Biol. 154:377-387; Stifani et al., 1992, Nature Genet. 2:119-127; Weinmaster et al., 1992, Development 116:931-941; Kopan et al., 1993, J. Cell Biol. 121:631-641; Lardelli et al., 1993, Exp. Cell Res. 204:364-372; Lardelli et al., 1994, Mech. Dev. 46:123-136; Henrique et al., 1995, 20 Nature 375:787-790; Horvitz et al., 1991, Nature 351:535-541; Franco del Amo et al., 1992, Development 115:737-744). Among the tissues in which mammalian Notch homologues are first expressed are the pre-somitic mesoderm and the developing neuroepithelium of the embryo. In the pre-somitic mesoderm, expression of Notch1 is seen in all of the migrated mesoderm, 25 and a particularly dense band is seen at the anterior edge of pre-somitic mesoderm. This expression has been shown to decrease once the somites have formed, indicating a role for Notch in the differentiation of somatic precursor cells (Reaume et al., 1992, Dev. Biol. 154:377-387; Horvitz et al., 30 1991, Nature 351:535-541). Similar expression patterns are seen for mouse Delta (Simske et al., 1995, Nature 375:142-145).

Within the developing mammalian nervous system, expression patterns of Notch homologue have been shown to be prominent in particular regions of the ventricular zone of the spinal cord, as well as in components of the peripheral nervous system, in an overlapping but non-identical pattern. Notch expression in the nervous system appears to be limited to regions of cellular proliferation, and is absent from nearby populations of recently differentiated cells (Weinmaster et al., 1991, *Development* 113:199-205; Reaume et al., 1992, *Dev. Biol.* 154:377-387; Weinmaster et al., 1992, ¹⁰ *Development* 116:931-941; Kopan et al., 1993, *J. Cell Biol.* 121:631-641; Lardelli et al., 1993, *Exp. Cell Res.* 204:364-372; Lardelli et al., 1994, *Mech. Dev.* 46:123-136; Henrique et al., 1995, *Nature* 375:787-790; Horvitz et al., 1991, *Nature* 351:535-541). A rat Notch ligand is also ¹⁵ expressed within the developing spinal cord, in distinct bands of the ventricular zone that overlap with the expression domains of the Notch genes. The spatio-temporal expression pattern of this ligand correlates well with the patterns of cells committing to spinal cord neuronal fates, ²⁰ which demonstrates the usefulness of Notch as a marker of populations of cells for neuronal fates (Henrique et al., 1995, *Nature* 375:787-790). This has also been suggested for vertebrate Delta homologues, whose expression domains also overlap with those of Notch1 (Larsson et al., 1994, *Genomics* 24:253-258; Fortini et al., 1993, *Nature* 365:555-557; Simske ²⁵ et al., 1995, *Nature* 375:142-145). In the cases of the *Xenopus* and chicken homologues, Delta is actually expressed only in scattered cells within the Notch1 expression domain, as would be expected from the lateral specification model, and these patterns "foreshadow" future patterns of neuronal ³⁰ differentiation (Larsson et al., 1994, *Genomics* 24:253-258; Fortini et al., 1993, *Nature* 365:555-557).

Other vertebrate studies of particular interest have focused on the expression of Notch homologues in developing sensory structures, including the retina, hair follicles and tooth buds. In the case of the *Xenopus* retina, 5 Notch1 is expressed in the undifferentiated cells of the central marginal zone and central retina (Coffman et al., 1990, *Science* 249:1439-1441; Mango et al., 1991, *Nature* 352:811-815). Studies in the rat have also demonstrated an association of Notch1 with differentiating cells in the developing retina have been interpreted to suggest that 10 Notch1 plays a role in successive cell fate choices in this tissue (Lyman et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:10395-10399).

A detailed analysis of mouse Notch1 expression in the regenerating matrix cells of hair follicles was 15 undertaken to examine the potential participation of Notch proteins in epithelial/mesenchymal inductive interactions (Franco del Amo et al., 1992, *Development* 115:737-744). Such a role had originally been suggested for Notch1 based on the its expression in rat whiskers and tooth buds (Weinmaster et 20 al., 1991, *Development* 113:199-205). Notch1 expression was instead found to be limited to subsets of non-mitotic, differentiating cells that are not subject to epithelial/mesenchymal interactions, a finding that is consistent with Notch expression elsewhere.

25 Expression studies of Notch proteins in human tissue and cell lines have also been reported. The aberrant expression of a truncated Notch1 RNA in human T-cell leukemia results from a translocation with a breakpoint in Notch1 (Ellisen et al., 1991, *Cell* 66:649-661). A study of human Notch1 expression during hematopoiesis has suggested a role 30 for Notch1 in the early differentiation of T-cell precursors (Mango et al., 1994, *Development* 120:2305-2315). Additional studies of human Notch1 and Notch2 expression have been

performed on adult tissue sections including both normal and neoplastic cervical and colon tissue. Notch1 and Notch2 appear to be expressed in overlapping patterns in differentiating populations of cells within squamous 5 epithelia of normal tissues that have been examined and are clearly not expressed in normal columnar epithelia, except in some of the precursor cells. Both proteins are expressed in neoplasias, in cases ranging from relatively benign squamous metaplasias to cancerous invasive adenocarcinomas in which columnar epithelia are replaced by these tumors (Mello et 10 al., 1994, *Cell* 77:95-106).

Insight into the developmental role and the general nature of Notch signaling has emerged from studies with truncated, constitutively activated forms of Notch in several species. These recombinantly engineered Notch forms, which 15 lack extracellular ligand-binding domains, resemble the naturally occurring oncogenic variants of mammalian Notch proteins and are constitutively activated using phenotypic criteria (Greenwald, 1994, *Curr. Opin. Genet. Dev.* 4:556; Fortini et al., 1993, *Nature* 365:555-557; Coffman et al., 20 1993, *Cell* 73:659-671; Struhl et al., 1993, *Cell* 69:1073; Rebay et al., 1993, *Genes Dev.* 7:1949; Kopan et al., 1994, *Development* 120:2385; Roehl et al., 1993, *Nature* 364:632).

- Ubiquitous expression of activated Notch in the *Drosophila* embryo suppresses neuroblast segregation without impairing epidermal differentiation (Struhl et al., 1993, 25 *Cell* 69:331; Rebay et al., 1993, *Genes Dev.* 7:1949).

- Persistent expression of activated Notch in developing imaginal epithelia likewise results in an overproduction of epidermis at the expense of neural structures (Struhl et al., 1993, *Cell* 69:331).

30 - Neuroblast segregation occurs in temporal waves that are delayed but not prevented by transient expression of

activated Notch in the embryo (Struhl et al., 1993, *Cell* 69:331).

- Transient expression in well-defined cells of the *Drosophila* eye imaginal disc causes the cells to ignore their 5 normal inductive cues and to adopt alternative cell fates (Fortini et al., 1993, *Nature* 365:555-557).

- Studies utilizing transient expression of activated Notch in either the *Drosophila* embryo or the eye disc indicate that once Notch signaling activity has subsided, 10 cells may recover and differentiate properly or respond to later developmental cues (Fortini et al., 1993, *Nature* 365:555-557; Struhl et al., 1993, *Cell* 69:331).

For a general review on the Notch pathway and Notch signaling, see Artavanis-Tsakonas et al., 1995, *Science* 268:225-232.

15 Ligands, cytoplasmic effectors and nuclear elements of Notch signaling have been identified in *Drosophila*, and vertebrate counterparts have also been cloned (reviewed in Artavanis-Tsakonas et al., 1995, *Science* 268:225-232). While protein interactions between the various elements have been 20 documented, the biochemical nature of Notch signaling remains elusive. Expression of truncated forms of Notch reveal that Notch proteins without transmembrane and extracellular domains are translocated to the nucleus both in transgenic flies and in transfected mammalian or *Drosophila* cells (Lieber et al., 1993, *Genes and Development* 7:1949-1965; 25 Fortini et al., 1993, *Nature* 365:555-557; Ahmad et al., 1995, *Mechanisms of Development* 53:78-85; Zagouras et al., 1995, *Proc. Natl. Acad. Sci. USA* 92:6414-6418). Sequence 30 comparisons between mammalian and *Drosophila* Notch molecules, along with deletion analysis, have found two nuclear localization sequences that reside on either side of the Ankyrin repeats (Stifani et al., 1992, *Nature Genetics* 2:119-127; Lieber et al., 1993, *Genes and Development* 7:1949-1965;

Kopan et al., 1994, Development 120:2385-2396). These findings prompted the speculation that Notch may be directly participating in nuclear events by means of a proteolytic cleavage and subsequent translocation of the intracellular 5 fragment into the nucleus. However, conclusive functional evidence for such a hypothesis remains elusive (Artavanis-Tsakonas et al., 1995, Science 268:225-232).

10 Citation or identification of any reference in Section 2 or any other section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

15 The inventors have discovered that Delta is cleaved by the metalloprotease-disintegrin Kuzbanian (Kuz) into two fragments, a soluble amino-terminal fragment consisting essentially of the extracellular domain, and a membrane-bound fragment consisting essentially of the transmembrane domain and the intracellular domain. The soluble fragment of Delta, like the full length, membrane-bound Delta, is able to bind 20 to Notch. Although not intending to be limited to any particular mechanism, Applicants believe that even though full length Delta is able to bind to Notch, it is the soluble fragment of Delta that is the actual ligand for Notch *in vivo*.

25 The detection or measurement of Delta activation, i.e., cleavage, is important in the study and manipulation of differentiation processes, since Delta plays a key role in cell fate (differentiation) determination, and since Delta is a ligand of Notch, Notch also playing a key role in cell fate (differentiation) determination. Molecules that modulate 30 Delta and Notch function are important tools for studying and manipulating differentiation processes, e.g., in expanding cell populations without substantial differentiation

(International Publication WO 97/11716), in cancer studies and therapy (International Publication WO 94/07474), and differentiation studies on normal tissue. Molecules that allow the detection or measurement of Notch or Delta mRNA or 5 protein levels or activity also have use in studying and manipulating differentiation processes. Accordingly, molecules that can be used to generate or detect anti-Delta antibodies or Delta nucleic acids have use in such detection or measurement.

One embodiment of the present invention is directed 10 to a peptide of approximately 30 amino acids, and its encoding nucleic acids, of the toporythmic protein Delta that contains a sequence which is cleaved by the metalloprotease-disintegrin Kuzbanian (Kuz), (herein termed "cleavage peptide") as well as derivatives (e.g., fragments) and 15 analogs thereof. For example, the Delta cleavage peptide consists of the sequence of amino acid Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), of amino acid Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), of amino acid Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), of 20 amino acid Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), and the sequence of amino acid Cys₅₆₄ to amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). Nucleic acids hybridizable to or complementary to the cleavage peptide encoding nucleic acids are also provided. In a 25 specific embodiment, the Delta cleavage peptide is a portion of a mammalian Delta, preferably a human Delta. Such a peptide is believed to have the ability to modulate Kuz cleavage of Delta, and thus, Delta and Notch activation.

In a specific embodiment, the present invention is directed to a peptide comprising a fragment of a Delta 30 protein, the amino acid sequence of the peptide consisting of the amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse

Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). In another embodiment, a 5 fragment of a Delta protein of not more than 150 or 50 or 30 amino acids comprising a Delta sequence selected from the group consisting of amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in 10 *Xenopus* Delta (SEQ ID NO:8), and Cys₅₆₄ to amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). In yet another embodiment, the invention is directed to a peptide the amino acid sequence of which consists of amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to 15 amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9).

The invention is also directed to a derivative or 20 analog of the cleavage peptide which is functionally active, i.e., capable of displaying one or more known functional activities associated with the "wild type" cleavage peptide. Such functional activities include but are not limited to antigenicity [ability to bind (or compete with the cleavage 25 peptide for binding) to an anti-Delta cleavage peptide antibody], immunogenicity (ability to generate antibody which binds to the cleavage peptide), ability to bind (or compete with the cleavage peptide for binding) to Kuz. The invention is further directed to a fragment (and derivatives or analogs thereof) of the Delta cleavage peptide which is able to bind 30 to Kuz.

Antibodies to the Delta cleavage peptide, its derivatives and analogs, are additionally provided.

Delta fragments that comprise the cleavage peptide sequence are also provided, as are fusion proteins comprising a Delta fragment containing a sequence of Delta that includes at least the cleavage peptide sequence, fused to a non-Delta sequence at the amino- and/or carboxy-terminal end of the Delta sequence. Concatamers of Delta fragments containing at least the cleavage peptide sequence (e.g., two, three, or more copies of a portion of the Delta sequence consisting of at least the cleavage peptide sequence) are also provided.

In particular embodiments, the Delta fragments comprising the cleavage peptide sequence are not greater than 35, 50, 75, 100, 150, or 200 amino acids in length. In a specific embodiment, the present invention is directed to a chimeric protein comprising a Delta protein sequence fused to a non-Delta protein sequence, wherein the Delta protein sequence is a sequence of not more than 100 or 50 or 30 amino acids that comprises the amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in Xenopus Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ or Gln₅₉₄ in Drosophila Delta (SEQ ID NO:9).

In another embodiment, the present invention is directed to a peptide comprising an amino-terminal fragment of a full length Delta protein, which fragment is cleaved from the full length Delta protein by two proteolytic processing events, the cleavage of the signal peptide and the cleavage by Kuz, (herein termed "soluble Delta peptide" or "D¹EC") as well as derivatives and analogs thereof. For example, the soluble Delta peptide amino acid sequence begins at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); begins at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); begins at

amino acid Ser₂₄ and terminates between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), begins at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or begins at 5 amino acid Ser₂₃ and terminates between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). Such a peptide is believed to have the ability to bind Notch, and thus modulate Delta and Notch activation.

The invention is also directed to a derivative or 10 analog of the soluble Delta peptide which is functionally active, i.e., capable of displaying one or more known functional activities associated with the "wild type" soluble peptide. Such functional activities include but are not limited to antigenicity [ability to bind (or compete with the soluble peptide for binding) to an anti-Delta soluble peptide 15 antibody], immunogenicity (ability to generate antibody which binds to the soluble peptide), ability to bind (or compete with the soluble peptide for binding) to Notch.

Antibodies to the Delta soluble peptide, its derivatives and analogs, are additionally provided.

20 Methods of production of the Delta cleavage peptide, derivatives and analogs, e.g., by recombinant means, are also provided. Methods of production of the soluble Delta peptide, derivatives and analogs, e.g., by recombinant means, are also provided.

25 The present invention is also directed to certain compositions comprising and methods for production of protein complexes of Delta and Kuz. Specifically, in this embodiment, the invention is directed to complexes of Delta, and derivatives, fragments and analogs of Delta, with Kuz, and its derivatives, fragments and analogs (a complex of 30 Delta and Kuz is designated as "Delta:Kuz" herein). Methods of production of a Delta:Kuz complex, and a derivative or

analog thereof, e.g., by recombinant means, are also provided.

The present invention is also directed to certain compositions comprising and methods for production of protein complexes of Notch and a soluble fragment of Delta consisting essentially of the extracellular domain that is liberated by the proteolytic processing of Delta by Kuz ("soluble Delta peptide" or "Dl^{EC}"). Specifically, in this embodiment, the invention is directed to complexes of the soluble Delta peptide, and derivatives, fragments and analogs of the soluble Delta peptide, with Notch, and its derivatives, fragments and analogs (a complex of the soluble fragment of Delta and Notch is designated as "Dl^{EC}:Notch" herein). Methods of production of a Dl^{EC}:Notch complex, and a derivative or analog thereof, e.g., by recombinant means, are also provided.

The invention is further directed to methods for modulating (i.e., inhibiting or enhancing) the activity of Notch or Delta or Kuz by contacting a cell expressing Notch or Delta or Kuz, or an organism comprising a cell expressing Notch or Delta or Kuz, a peptide comprising a fragment of Delta having the amino acid sequence of about amino acid Cys₅₁₆ to about amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), of about amino acid Cys₅₁₅ to about amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), of about amino acid Cys₅₂₃ to about amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), of about amino acid Cys₅₁₈ to about amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), and the sequence of about amino acid Cys₅₆₄ to about amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). In specific embodiments, the peptide comprises 25, 30, 35, 40, 50, 100, 150, 200 or 250 amino acids of Delta.

The invention is further directed to methods for modulating (i.e., inhibiting or enhancing) the activity of Notch or Delta or Kuz or at least one of their signalling

pathways by contacting a cell or organism expressing Notch or Delta or Kuz with a peptide comprising a fragment of a Delta protein having the amino acid sequence beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); and the sequence beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9).

The invention is further directed to methods for modulating (i.e., inhibiting or enhancing) the activity of a Delta:Kuz complex or a Dl^{EC}:Notch complex. The protein components of a Delta:Kuz complex or a Dl^{EC}:Notch complex have been implicated in cell fate and differentiation.

Accordingly, the present invention is directed to methods for screening a Delta:Kuz complex, as well as a derivative or analog of the complex, for the ability to alter cell fate or differentiation. The present invention is also directed to methods for screening a Dl^{EC}:Notch complex, as well as a derivative or analog of the complex, for the ability to alter cell fate or differentiation.

The present invention is also directed to therapeutic and diagnostic methods and compositions based on the Delta cleavage peptide and encoding nucleic acids, as well as on soluble Delta peptide and encoding nucleic acids. The invention provides for the treatment of disorders of cell fate and differentiation by administration of a therapeutic compound of the invention. Such therapeutic compounds (termed herein "Therapeutics") include: Delta cleavage

peptides and derivative and analogs (including fragments) thereof, antibodies thereto, nucleic acids encoding the Delta cleavage peptide, derivatives, or analogs, Delta cleavage peptide antisense nucleic acids, Delta:Kuz complexes and 5 antibodies thereto, and D^{1_{EC}}:Notch complexes and antibodies thereto. In addition, such Therapeutics include soluble Delta peptides and derivatives and analogs thereof, antibodies thereto, nucleic acids encoding the soluble Delta peptides, derivatives, or analogs, and soluble Delta peptide antisense nucleic acids. In a preferred embodiment, a 10 Therapeutic of the invention is administered to treat a cancerous condition, or to prevent progression from a pre-neoplastic or non-malignant state into a neoplastic or a malignant state. In other specific embodiments, a Therapeutic of the invention is administered to treat a 15 nervous system disorder or to promote tissue regeneration and repair.

In one embodiment, Therapeutics which antagonize, or inhibit, Notch, Delta cleavage peptide and/or Kuz function (hereinafter "Antagonist Therapeutics") are administered for 20 therapeutic effect. In another embodiment, Therapeutics which promote Notch, Delta cleavage peptide and/or Kuz function (hereinafter "Agonist Therapeutics") are administered for therapeutic effect.

Disorders of cell fate, in particular 25 hyperproliferative (e.g., cancer) or hypoproliferative disorders, involving aberrant or undesirable levels of expression or activity or localization of Notch, Delta cleavage peptide and or Kuz protein can be diagnosed by detecting such levels, as described more fully *infra*.

Yet another embodiment of the present invention is 30 directed to methods for detecting or measuring Delta activation by observing or measuring Delta cleavage products that are indicative of Delta activation. In one aspect of

this embodiment of the invention, the method for detecting or measuring Delta activation in a cell comprises detecting or measuring the expression of one or more Delta cleavage products selected from the group consisting of Dl^{EC} and DlTM.

5 In yet another aspect, the method comprises detecting or measuring an amino-terminal fragment of full-length Delta terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9), between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta

10 (SEQ ID NO:6), between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), or terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8). In yet another aspect, the method comprises detecting or measuring under reducing conditions, a soluble Delta

15 fragment of approximately 67 kilodaltons (Dl^{EC}). In yet another aspect, the method comprises detecting or measuring a soluble Delta peptide having the amino acid sequence beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7); beginning at amino acid Ser₂₂ and terminating between

20 amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); and the sequence beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9).

The present invention is also directed to methods for detecting or measuring Kuz function by observing or

30 measuring Delta cleavage products that are indicative of Kuz function. In one aspect of this embodiment of the invention, the method for detecting or measuring Kuz function in a cell

comprises detecting or measuring the expression of one or more Delta cleavage products selected from the group consisting of Dl^{EC} and DlTM. In yet another aspect, the method comprises detecting or measuring an amino-terminal fragment 5 of full-length Delta which terminates between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta, between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta, between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta, between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta, or terminates between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ 10 in *Xenopus* Delta. In yet another aspect, the method comprises detecting or measuring under reducing conditions, a soluble Delta fragment of approximately 67 kilodaltons. In yet another aspect, the method comprises detecting or measuring a soluble Delta peptide having the amino acid 15 sequence beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); beginning at amino acid Ser₂₄ and terminating between 20 amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); and the sequence beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or 25 Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9).

In another embodiment, the present invention is also directed to methods for identifying a molecule that modulates Delta activation by detecting or measuring a change in the amount or pattern of Delta cleavage products. In one aspect of this embodiment of the invention, the method for 30 identifying a modulator of Delta activation comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or

more Delta cleavage products selected from the group consisting of Dl^{EC} and Dl^{TM} , in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with the candidate 5 molecule indicates that the molecule modulates Delta activity.

In an alternative aspect, the method for identifying a modulator of Delta activation comprises contacting a candidate modulator molecule with a full length 10 Delta in the presence of a composition comprising Kuz and optionally other cellular proteins, under conditions conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of the composition and detecting or measuring the amount of Delta cleavage products Dl^{EC} and Dl^{TM} that result, in which a difference in the 15 presence or amount of said Delta cleavage products compared to a full-length Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

In yet another embodiment, the present invention is 20 also directed to methods for identifying a molecule that modulates Kuz function by detecting or measuring a change in the amount of Delta cleavage products that are necessary for Kuz function. In one aspect of this embodiment of the invention, the method for identifying a modulator of Kuz 25 function comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more Delta cleavage products selected from the group consisting of Dl^{EC} and Dl^{TM} , in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with 30 the candidate molecule indicates that the molecule modulates Notch function.

In yet another embodiment, the present invention is also directed to methods for identifying a molecule that modulates Kuz function by detecting or measuring a change in the amount of Delta cleavage products that are indicative of 5 Kuz function. In one aspect of this embodiment of the invention, the method for identifying a modulator of Kuz function comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more Delta cleavage products selected 10 from the group consisting of Dl^{EC} and Dl^{TM} , in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Kuz function.

The present invention is also directed to 15 therapeutic and prophylactic, as well as diagnostic, prognostic, and screening methods and compositions based upon the Delta:Kuz complex or the Dl^{EC} :Notch complex (and the nucleic acids encoding the individual proteins that participate in the complex). Therapeutic compounds of the 20 invention include, but are not limited to, a Delta:Kuz complex, and a complex where one or both members of the complex is a derivative, fragment, homolog or analog of Delta or Kuz; antibodies to and nucleic acids encoding the foregoing; and antisense nucleic acids to the nucleotide 25 sequences encoding the complex components. Diagnostic, prognostic and screening kits are also provided.

Animal models and methods of screening for modulators (i.e., agonists, and antagonists) of the activity of a Delta:Kuz complex or of a Dl^{EC} :Notch complex are also provided.

30 Methods of identifying molecules that inhibit, or alternatively, that increase formation of a Delta:Kuz complex or of a Dl^{EC} :Notch complex are also provided.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic diagram of the Notch signaling pathway. The Notch receptor can bind to either Delta or Serrate through its extracellular domain. Ligand binding can result in receptor multimerization that is stabilized by interactions between the intracellular ankyrin repeats of Notch and the cytoplasmic protein Deltex. These events can control the nuclear translocation of the DNA-binding protein Suppressor of Hairless and its known association with the Hairless protein. The transcriptional induction of the Enhancer of Split basic helix-loop-helix (bHLH) genes appears to depend on Notch signaling.

Figure 2 is a Notch homolog sequence comparison. The human Notch2 (humN2) (SEQ ID NO:1), human Notch1 (humN1) (SEQ ID NO:2), *Xenopus* Notch/Xotch (XenN) (SEQ ID NO:3), and *Drosophila* Notch (DrosN) (SEQ ID NO:4) protein sequences are aligned, with names indicated to the left and numbering to the right (Wharton et al., 1985, Cell 43:567-581; Coffman et al., 1990, Science 249:1438-1441; Ellisen et al., 1991, Cell 66:649-661; Stifani et al., 1992, Nature Genetics 2:119-127). Major Notch protein motifs are enclosed in boxes. Starting from the N-terminal, the boxed regions indicate: EGF repeats, Lin-12/Notch (LN) repeats, transmembrane domain (TM), Ankyrin repeats, and PEST-containing region. Also indicated are the putative CcN motif components (Stifani et al., 1992, Nature Genetics 2:119-127) nuclear localization signal (NLS, BNTS) and putative CKII and cdc2 phosphorylation sites. The calculated signal cleavage site is indicated with an arrow.

Figure 3 is a Delta homolog sequence comparison. The human Delta (HDL) (SEQ ID NO:5), mouse Delta (MDL) (SEQ ID NO:6), chick Delta (CDL) (SEQ ID NO:7), *Xenopus* Delta (XDL) (SEQ ID NO:8), and *Drosophila* Delta (DDL) (SEQ ID NO:9) protein sequences are aligned, with names indicated to the

left and numbering to the right. Major Delta protein motifs are labeled.

Figure 4A and 4B is the amino acid sequence (SEQ ID NO:10) and the nucleic acid sequence (SEQ ID NO:11), 5 respectfully, of human Delta.

Figure 5A and 5B is the amino acid sequence (SEQ ID NO:12) and the nucleic acid sequence (SEQ ID NO:13), respectfully, of the human Kuz homolog.

Figures 6A-6F shows results of a genetic modifier screen that was carried out to identify genes that 10 genetically interact with *kuz*. A strain constitutively expressing a KuzDN construct in developing imaginal discs was used in the screen (expression of a KuzDN construct lacking the proprotein and metalloprotease domains was driven by a GAL4 line 32B) which causes adult mutant phenotypes, 15 including extra wing vein materials, mostly notably deltas at the ends of the longitudinal veins (denoted by arrowheads in Figure 6A), small and rough eyes, and extra bristles on the notum (denoted by arrowheads in Figure 6E). More than 2400 lethal P-element insertions were screened for phenotypic 20 modification effects on KuzDN. Seven P-insertions were found to cause significant reduction of the viability (semi-lethal) of the KuzDN flies when they are also heterozygous for each of the P-insertion. Preliminary characterization of these P- insertions revealed that two of them are *Kuz* alleles and one 25 is a loss-of-function Delta allele while the nature of the other insertions are unknown. Flies that carry an extra copy of the Delta gene (+/+/) with the KuzDN background (Figures 6B, 6F) show an almost complete suppression of the KuzDN phenotypes. (Figure 6C) An extra copy of Notch (+/+/) (Ramos et al., 1989, Genetics 123:337-348) alone has an 30 essentially normal phenotype (Figure 6C). Notch (+/+/) gives negligible suppression of the KuzDN phenotype in KuzDN flies (Figure 6D).

Figures 7A-7E show that a soluble fragment of Delta (Dl^{EC}) is released constitutively in S2 cells *in vivo*. Figure 7A: Expression of Delta (Dl) antigen in stably transfected S2 cells (Rebay, et al., 1991, Cell 67:687-699) is detected by SDS-PAGE and western blotting with monoclonal antibody 9B of reduced (+Bme) and non-reduced (-Bme) cell extracts (c) and culture media (m). A product consistent with full length Delta is clearly detectable in the cell extract (MW~ 83,000 Daltons non-reduced and 90,000 Daltons reduced). A significant amount of a product of greater mobility is seen in the media (MW~ 62,000 Daltons non-reduced and 67,000 Daltons reduced) that is consistent in size with the extracellular domain of Delta (estimated MW~ 65,000 Daltons) and is referred to as Dl^{EC} . A 40-fold higher affinity of the antibody was observed for the non-reduced versus reduced Delta and was compensated for by increased protein load (4X) and exposure times (10X) in the reduced samples. Figure 7B: Bands of the same mobility are seen in extracts of wild type Drosophila embryos (16hr). Note that 1, 3, 5 and 10 embryos loaded on the gel demonstrate that the antigen is barely detectable in a single embryo ("1") but becomes clearer with the greater number of embryos loaded ("10"). Figure 7C: Affinity purified Dl^{EC} migrates at MW~ 62,000 Daltons under reducing conditions and at MW~ 67,000 Daltons under non-reducing conditions on a coomassie blue-stained SDS-PAGE gel. Figure 7D: Schematic of the Drosophila Delta protein demonstrates the DSL domain (DSL), the epidermal growth factor like repeats (EGF) and the transmembrane domain (TM). Amino acid numbering of N-terminus, the beginning of the TM domain and the C-terminus is shown. Figure 7E: Thirteen cycles of N-terminal amino acid sequence analysis of Dl^{EC} is shown with alignment to the *Drosophila* (dDl), *Xenopus* (xDl) and human (hDl) Delta amino acid sequences. The arrow indicates the conserved serine residue in the position of the

N-terminus of Dl^{EC} and the potential site of signal peptide processing for Dl.

Figures 8A-8D shows that Kuz plays a direct role in Delta processing *in vivo* and *in vitro*. Figure 8A: The first 5 two panels (-): Expression of Delta and Dl^{EC} are apparent by western blotting with the 9B antibody in the cell pellet (c) and the medium (m) in S2 cells transiently transfected with full length Delta (Fehon, et al., 1990, Cell 61:523-534). The second two panels (Kuz): Cotransfection of S2 cells with 10 Kuz and Delta results in an increase in the Dl^{EC} fragment in the cell culture media (m) which correlates with an apparent decrease in Delta in the cell pellet (c). The third two panels (KuzDN): Cotransfection with dominant negative Kuz dramatically decreases the Dl^{EC} observed in the media (m) and corresponds with greater amounts of full length Delta in the 15 cell pellet (c). Figure 8B: Cotransfection of Kuz and KuzDN with Notch was done under identical experimental conditions as for Delta and western blotted with the 9C6 Notch intracellular domain antibody (Fehon, et al., 1990, Cell 61:523-534) demonstrates a negligible effect on the 20 processing of Notch as seen by the invariant levels of NTM, the constitutively processed form of Notch (Blaumueller et al., 1997, Cell 90:281-291). Figure 8C: The metalloprotease inhibitors EDTA and 1,10-phenanthroline inhibit the endogenous S2 cell proteolytic activity yielding Dl^{EC}. The 25 left panel demonstrates the accumulation of Dl^{EC} at various time points up to 60 minutes in the medium of S2 cells stably expressing full length Delta (Rebay, et al., 1991, Cell 67:687-699). The right panel shows the accumulation of Dl^{EC} at 60 minutes in the presence of EDTA (5, 10, 15 mM) and 1,10-phenanthroline (5, 10 mM). Relatively high 30 concentrations of the chelators were required to overcome the concentrations Ca²⁺ (~8.6 mM) and other metal ions in the media and serum. Higher concentrations of 1,10-

phenanthroline proved to alter cell morphology. Both of these reagents, which are well documented metalloprotease inhibitors, inhibit accumulation of Dl^{EC} in the media. Figure 8D: Delta processing is inhibited in Kuz -/- embryos. Nine 5 Kuz +/- and Kuz -/- embryos were identified by morphology and the extracts analyzed by SDS-PAGE and western blotting with antibody 9B. Dl^{EC} is absent in Kuz -/- embryos and demonstrates a higher level of full length Delta compared to Kuz +/- embryos.

Figures 9A-9C shows that Dl^{EC} binds to Notch, 10 competes for Notch-Delta interaction and acts as an agonist of the Notch pathway. Figure 9A: The Dl^{EC} fragment specifically binds to Notch expressing S2 cells and does not bind to S2 cells alone. Notch expressing S2 cells (lane 1, 2) incubated in the absence (lane 1) or presence (lane 2) of 15 Dl^{EC} (lane 6) were sedimented through a sucrose cushion and the extract was western blotted with antibody 9B. Dl^{EC} was prepared as a 5X concentrate of 16 hour culture media (Sang's M3) of 0.7mM CuSO₄ induced Delta-S2 cells. Notch-S2 and nontransfected S2 cells were induced with 0.7 mM CuSO₄ for 20 16hrs in media with 5% serum. The cells were collected by centrifugation and washed once in serum free media with 1% bovine serum albumin (BSA) and resuspended at 2x10⁶ cells/mL in M3, 1% BSA. 250 μ L of cells were added to 100 μ L of Dl^{EC} concentrate, raised to 500 μ L with M3, 1% BSA and incubated one hour at room temperature on a rocking table at five 25 oscillations per minute. The mixture was layered over a cushion of 20% sucrose, 20mM TRIS-HCl, 150mM NaCl, 2mM CaCl₂, 1% BSA, pH 7.4, in microfuge tubes that had previously been blocked with 1% BSA. The tubes were centrifuged at 14,000 rpm for 3 minutes and the supernatant aspirated. The cell 30 pellets were washed two times with cold serum free media without resuspension of the pellet. The pellet was then lysed and dissolved in SDS-PAGE sample buffer without β -

mercaptoethanol and boiled for five minutes. The proteins were resolved by SDS-PAGE and western blotting with the 9B antibody. Lane 3 and 4 show parallel incubations with S2 cells in the absence (lane 3) or presence (lane 4) of Dl^{EC}.

5 Figure 9B: Preincubation of Notch-S2 cells with Dl^{EC} concentrate reduces their subsequent rate of aggregation with Delta-S2 cells as measured turbidimetrically with transmitted light at 320nm. At the concentration shown (1X Dl^{EC}, closed circles), a 60% inhibition in the initial rate of aggregation was seen compared to control media concentrate (1X ΔECN, closed squares). The error bars show the standard deviation of the mean of triplicate determinations. Figure 9C shows the effect of Dl^{EC} on primary cultured cortical neurons in the representative images as labeled: (I) seven to ten days *in vitro* cortical neurons before treatment, (II) cultured in the presence of ΔECN media, (III) cultured in the presence of Dl^{EC} media, (IV) affinity purified Dl^{EC}, and (V) buffer control for purified Dl^{EC}. The graph represents the mean length of neurites per neuron. Each bar represents the mean \pm SEM of three separate experimental trials. Primary cortical neurons 10 exhibit multipolar morphology and the extensive neurite network in control cultures (I), cultures in the presence of ΔECN media (II) and buffer control of purified Dl^{EC} (V). Note the decrease in the mean neurite length per neuron and limited neurite branching in cultures treated with Dl^{EC} media 15 (III) and purified Dl^{EC} (IV). Scale bar = 50 μ m.

20 25

Figure 10 is a schematic diagram comparing the soluble fragment of Delta (Dl^{EC}) that is clipped by Kuz with Dls.

Figure 11 shows the amino acid sequence of the Delta cleavage peptide of *Drosophila* Delta (SEQ ID NO:9). 30 Bold arrows indicate potential cleavage sites identified by data from both C-terminal sequence analysis and LC/MS; dashed arrows indicate potential cleavage sites identified by only

one of the analyses. (A) indicates the alanine instead of the threonine reported by Vässin et al., 1987, EMBO J. 6:3431-3440.

5 5. DETAILED DESCRIPTION OF THE INVENTION

The inventors have discovered that Delta is cleaved by the metalloprotease-disintegrin Kuzbanian (Kuz) into two fragments, a soluble amino-terminal fragment consisting essentially of the extracellular domain, and a membrane-bound fragment consisting essentially of the transmembrane domain and the intracellular domain. The soluble fragment of Delta, like the full length, membrane-bound Delta, is able to bind to Notch. Although not intending to be limited to any particular mechanism, Applicants believe that even though full length Delta is able to bind to Notch, it is the soluble fragment of Delta that is the actual ligand for Notch *in vivo*.

The detection or measurement of Delta activation, i.e., cleavage, is important in the study and manipulation of differentiation processes, since Delta plays a key role in cell fate (differentiation) determination, and since Delta is a ligand of Notch, Notch also playing a key role in cell fate (differentiation) determination. Molecules that modulate Delta and Notch function are important tools for studying and manipulating differentiation processes, e.g., in expanding cell populations without substantial differentiation (International Publication WO 97/11716), in cancer studies and therapy (International Publication WO 94/07474), and differentiation studies on normal tissue. Molecules that allow the detection or measurement of Notch or Delta mRNA or protein levels or activity also have use in studying and manipulating differentiation processes. Accordingly, molecules that can be used to generate or detect anti-Delta

antibodies or Delta nucleic acids have use in such detection or measurement.

One embodiment of the present invention is directed to a peptide of approximately 30 amino acids, and its encoding nucleic acids, of the toporythmic protein Delta that contains a sequence which is cleaved by the metalloprotease-disintegrin Kuzbanian (Kuz), (herein termed "cleavage peptide") as well as derivatives (e.g., fragments) and analogs thereof. For example, the Delta cleavage peptide consists of the sequence of amino acid Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta, of amino acid Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta, of amino acid Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta, of amino acid Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta, and the sequence of amino acid Cys₅₆₄ to amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta. Nucleic acids hybridizable to or complementary to the cleavage peptide encoding nucleic acids are also provided. In a specific embodiment, the Delta cleavage peptide is a portion of a mammalian Delta, preferably a human Delta. Such a peptide is believed to have the ability to modulate Kuz cleavage of Delta, and thus, Delta and Notch activation.

The invention is also directed to a derivative or analog of the cleavage peptide which is functionally active, i.e., capable of displaying one or more known functional activities associated with the "wild type" cleavage peptide. Such functional activities include but are not limited to antigenicity [ability to bind (or compete with the cleavage peptide for binding) to an anti-Delta cleavage peptide antibody], immunogenicity (ability to generate antibody which binds to the cleavage peptide), ability to bind (or compete with the cleavage peptide for binding) to Kuz. The invention is further directed to a fragment (and derivatives or analogs thereof) of the Delta cleavage peptide which is able to bind to Kuz.

Antibodies to the Delta cleavage peptide, its derivatives and analogs, are additionally provided.

Delta fragments that comprise the cleavage peptide sequence are also provided, as are fusion proteins comprising 5 a Delta fragment containing a sequence of Delta that includes at least the cleavage peptide sequence, fused to a non-Delta sequence at the amino- and/or carboxy-terminal end of the Delta sequence. Concatamers of Delta fragments containing at least the cleavage peptide sequence (e.g., two, three, or 10 more copies of a portion of the Delta sequence consisting of at least the cleavage peptide sequence) are also provided. In particular embodiments, the Delta fragments comprising the cleavage peptide sequence are not greater than 35, 50, 75, 100, 150, or 200 amino acids in length.

Methods of production of the Delta cleavage 15 peptide, derivatives and analogs, e.g., by recombinant means, are also provided.

In another embodiment, the present invention is directed to a peptide comprising an amino-terminal fragment of a full length Delta protein, which fragment is cleaved 20 from the full length Delta protein by two proteolytic processing events, the cleavage of the signal peptide and the cleavage by Kuz (herein termed "soluble Delta peptide" or "D¹^{EC}") as well as derivatives and analogs thereof. For example, the soluble Delta peptide amino acid sequence begins 25 at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); begins at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); begins at amino acid Ser₂₄ and terminates between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), begins at 30 amino acid Ser₂₂ and terminates between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in Xenopus Delta (SEQ ID NO:8), or begins at amino acid Ser₂₃ and terminates between amino acid Cys₅₆₄ and

amino acid Ala_{593} or Gln_{594} in *Drosophila Delta* (SEQ ID NO:9). Such a peptide is believed to have the ability to bind Notch, and thus modulate Delta and Notch activation.

The invention is also directed to a derivative or 5 analog of the soluble Delta peptide which is functionally active, i.e., capable of displaying one or more known functional activities associated with the "wild type" soluble peptide. Such functional activities include but are not limited to antigenicity [ability to bind (or compete with the 10 soluble peptide for binding) to an anti-Delta soluble peptide antibody], immunogenicity (ability to generate antibody which binds to the soluble peptide), ability to bind (or compete with the soluble peptide for binding) to Notch.

Antibodies to the Delta soluble peptide, its derivatives and analogs, are additionally provided.

15 Methods of production of the soluble Delta peptide, derivatives and analogs, e.g., by recombinant means, are also provided.

The present invention is also directed to certain compositions comprising and methods for production of protein 20 complexes of Delta and Kuz. Specifically, in this embodiment, the invention is directed to complexes of Delta, and derivatives, fragments and analogs of Delta, with Kuz, and its derivatives, fragments and analogs (a complex of Delta and Kuz is designated as "Delta:Kuz" herein). Methods 25 of production of a Delta:Kuz complex, and a derivative or analog thereof, e.g., by recombinant means, are also provided.

The present invention is also directed to certain compositions and methods for production of protein complexes with Notch of the soluble fragment of Delta liberated by Kuz. 30 Specifically, in this embodiment, the invention is directed to complexes of the soluble Delta peptide, and derivatives, fragments and analogs of the soluble fragment, with Notch,

and its derivatives, fragments and analogs (a complex of the soluble fragment of Delta and Notch is designated as "Dl^{EC}:Notch" herein). Methods of production of a Dl^{EC}:Notch complex, and a derivative or analog thereof, e.g., by 5 recombinant means, are also provided.

The invention is further directed to methods for modulating (i.e., inhibiting or enhancing) the activity of Notch or Delta or Kuz by contacting a cell expressing Notch or Delta or Kuz, or an organism comprising a cell expressing 10 Notch or Delta or Kuz, a peptide comprising a fragment of Delta having the amino acid sequence of about amino acid Cys₅₁₆ to about amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), of about amino acid Cys₅₁₅ to about amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), of about amino acid Cys₅₂₃ to 15 about amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), of about amino acid Cys₅₁₈ to about amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), and the sequence of about amino acid Cys₅₆₄ to about amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). In specific embodiments, the peptide comprises 25, 30, 35, 40, 50, 100, 150, 200 or 250 amino acids of Delta. 20

The invention is further directed to methods for modulating (i.e., inhibiting or enhancing) the activity of Notch or Delta or Kuz or at least one of their signalling pathways by contacting a cell or organism expressing Notch or Delta or Kuz with a peptide comprising a fragment of a Delta 25 protein having the amino acid sequence beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino 30 acid Phe₅₅₁ in chick Delta (SEQ ID NO:7); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); and the sequence

beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9).

The invention is further directed to methods for modulating (i.e., inhibiting or enhancing) the activity of a Delta:Kuz complex or the activity of a Dl^{EC}:Notch complex. The protein components of a Delta:Kuz complex and of a Dl^{EC}:Notch complex have been implicated in cell fate and differentiation. Accordingly, the present invention is directed to methods for screening a Delta:Kuz complex, as well as a derivative or analog of the complex, for the ability to alter cell fate or differentiation. The present invention is also directed to methods for screening a Dl^{EC}:Notch complex, as well as a derivative or analog of the complex, for the ability to alter cell fate or differentiation.

The present invention is also directed to therapeutic and diagnostic methods and compositions based on the Delta cleavage peptide and encoding nucleic acids, as well as on soluble Delta peptides and encoding nucleic acids. The invention provides for the treatment of disorders of cell fate and differentiation by administration of a therapeutic compound of the invention. Such therapeutic compounds (termed herein "Therapeutics") include: Delta cleavage peptides and derivative and analogs (including fragments) thereof, antibodies thereto, nucleic acids encoding the Delta cleavage peptide, derivatives, or analogs, Delta cleavage peptide antisense nucleic acids, Delta:Kuz complexes and antibodies thereto, and Dl^{EC}:Notch complexes and antibodies thereto. In addition, such Therapeutics include soluble Delta peptides and derivatives and analogs thereof, antibodies thereto, nucleic acids encoding the soluble Delta peptides, derivatives, or analogs, and soluble Delta peptide antisense nucleic acids. In a preferred embodiment, a

Therapeutic of the invention is administered to treat a cancerous condition, or to prevent progression from a pre-neoplastic or non-malignant state into a neoplastic or a malignant state. In other specific embodiments, a

5 Therapeutic of the invention is administered to treat a nervous system disorder or to promote tissue regeneration and repair.

In one embodiment, Therapeutics which antagonize, or inhibit, Notch, Delta cleavage peptide and/or Kuz function (hereinafter "Antagonist Therapeutics") are administered for therapeutic effect. In another embodiment, Therapeutics which promote Notch, Delta cleavage peptide and/or Kuz function (hereinafter "Agonist Therapeutics") are administered for therapeutic effect.

Disorders of cell fate, in particular

15 hyperproliferative (e.g., cancer) or hypoproliferative disorders, involving aberrant or undesirable levels of expression or activity or localization of Notch, Delta cleavage peptide and or Kuz protein can be diagnosed by detecting such levels, as described more fully *infra*.

20 Yet another embodiment of the present invention is directed to methods for detecting or measuring Delta activation by observing or measuring Delta cleavage products that are indicative of Delta activation. In one aspect of this embodiment of the invention, the method for detecting or

25 measuring Delta activation in a cell comprises detecting or measuring the expression of one or more Delta cleavage products selected from the group consisting of D₁^{EC} and D₁TM. In yet another aspect, the method comprises detecting or measuring an amino-terminal fragment of full-length Delta terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃, or

30 Gln₅₉₄ in *Drosophila* Delta, between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta, between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta, between amino acid Cys₅₂₃ and amino

acid Phe₅₅₁ in chick Delta, or terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta. In yet another aspect, the method comprises detecting or measuring under reducing conditions, a soluble Delta fragment of 5 approximately 67 kilodaltons. In yet another aspect, the method comprises detecting or measuring a soluble Delta peptide having the amino acid sequence beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); and the sequence 10 beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). 15

The present invention is also directed to methods for detecting or measuring Kuz function by observing or 20 measuring Delta cleavage products that are indicative of Kuz function. In one aspect of this embodiment of the invention, the method for detecting or measuring Kuz function in a cell comprises detecting or measuring the expression of one or more Delta cleavage products selected from the group 25 consisting of D1^{EC} and D1TM. In yet another aspect, the method comprises detecting or measuring an amino-terminal fragment of full-length Delta which terminates between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta, between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta, between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta, 30 between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta, or terminates between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta. In yet another aspect, the method

comprises detecting or measuring under reducing conditions, a soluble Delta fragment of approximately 67 kilodaltons. In yet another aspect, the method comprises detecting or measuring a soluble Delta peptide having the amino acid sequence beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); and the sequence beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9).

15 In another embodiment, the present invention is also directed to methods for identifying a molecule that modulates Delta activation by detecting or measuring a change in the amount or pattern of Delta cleavage products. In one aspect of this embodiment of the invention, the method for identifying a modulator of Delta activation comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more Delta cleavage products selected from the group consisting of Dl^{EC} and DlTM, in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

20 In an alternative aspect, the method for identifying a modulator of Delta activation comprises contacting a candidate modulator molecule with a full length Delta in the presence of a composition comprising Kuz and optionally other cellular proteins, under conditions

conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of the composition and detecting or measuring the amount of Delta cleavage products Dl^{EC} and DlTM that result, in which a difference in the 5 presence or amount of said Delta cleavage products compared to a full-length Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

In yet another embodiment, the present invention is 10 also directed to methods for identifying a molecule that modulates Notch function by detecting or measuring a change in the amount of Delta cleavage products that are necessary for Notch function. In one aspect of this embodiment of the invention, the method for identifying a modulator of Notch function comprises providing a cell with a candidate 15 modulator molecule and detecting or measuring the expression by the cell of one or more Delta cleavage products selected from the group consisting of Dl^{EC} and DlTM, in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with 20 the candidate molecule indicates that the molecule modulates Notch function.

In yet another embodiment, the present invention is 25 also directed to methods for identifying a molecule that modulates Kuz function by detecting or measuring a change in the amount of Delta cleavage products that are indicative of Kuz function. In one aspect of this embodiment of the invention, the method for identifying a modulator of Kuz function comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more Delta cleavage products selected 30 from the group consisting of Dl^{EC} and DlTM, in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with

the candidate molecule indicates that the molecule modulates Kuz function.

The present invention is also directed to therapeutic and prophylactic, as well as diagnostic, 5 prognostic, and screening methods and compositions based upon the Delta:Kuz complex or a Dl^{EC}:Notch complex (and the nucleic acids encoding the individual proteins that participate in the complex). Therapeutic compounds of the invention include, but are not limited to, a Delta:Kuz complex, and a complex where one or both members of the complex is a 10 derivative, fragment, homolog or analog of Delta or Kuz; antibodies to and nucleic acids encoding the foregoing; and antisense nucleic acids to the nucleotide sequences encoding the complex components. Diagnostic, prognostic and screening kits are also provided.

15 Animal models and methods of screening for modulators (i.e., agonists, and antagonists) of the activity of a Delta:Kuz complex or the activity of a Dl^{EC}:Notch complex are also provided.

Methods of identifying molecules that inhibit, or 20 alternatively, that increase formation of a Delta:Kuz complex or of a Dl^{EC}:Notch complex are also provided.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

25

5.1 DELTA CLEAVAGE PEPTIDES, SOLUBLE DELTA PEPTIDES AND DELTA:KUZ PROTEIN COMPLEXES

5.1.1 DELTA CLEAVAGE PEPTIDES AND SOLUBLE DELTA PEPTIDES

Delta encoding nucleic acids from both vertebrate 30 and non-vertebrate species have been cloned, see e.g., International Patent Publication WO 97/01571 for a description of vertebrate, including human, Delta encoding nucleic acids. Human Delta encoding sequences and the

encoded amino acid sequence is available in GenBank under Accession No. AF003522 and are depicted in Figures 4A and 4B. The nucleotide sequence coding for a Delta cleavage peptide, or for a soluble Delta peptide, or a functionally active 5 fragment or other derivative thereof, can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native *Delta* gene and/or its flanking 10 regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such 15 as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation 20 elements may be used. In a specific embodiment, the human Delta cleavage peptide is expressed. In another specific embodiment, the human soluble Delta peptide is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to 25 construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a Delta cleavage 30 peptide or peptide fragment thereof may be regulated by a second nucleic acid sequence so that the Delta cleavage peptide is expressed in a host transformed with the

recombinant DNA molecule. For example, expression of a Delta cleavage peptide may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control Delta cleavage peptide expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Kamaroff, et al., 1978, *Proc. Natl. Acad. Sci. U.S.A.* 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:21-25); see also "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., *Nature* 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, *Nucl. Acids Res.* 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, *Nature* 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, *Cell* 38:639-646; Ornitz et al., 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409; MacDonald, 1987, *Hepatology* 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-122), immunoglobulin gene control region

which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in 5 testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-10 antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region 15 which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropin releasing hormone gene control region which is active in the 20 hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Expression vectors containing inserts of nucleic acids encoding a Delta cleavage peptide or encoding a soluble Delta peptide can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted 25 sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to the inserted Delta cleavage peptide coding sequences. In the second approach, the recombinant 30 vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics,

transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the Delta cleavage peptide encoding nucleic acids are inserted within the marker gene 5 sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on 10 the physical or functional properties of the encoded cleavage peptide in *in vitro* assay systems, e.g., binding to Kuz, binding with antibody.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and 15 growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as 20 vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which 25 modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered Delta cleavage peptide may be controlled. Furthermore, different host cells 30 have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage [e.g., of signal

sequence]) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an 5 unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous mammalian Delta cleavage peptide, or to ensure "native" glycosylation of a heterologous mammalian soluble 10 Delta peptide. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

In other specific embodiments, the Delta cleavage peptide, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the peptide, fragment, analog, or derivative joined via a peptide 15 bond to a heterologous protein sequence (of a different protein)). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric 20 product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

One embodiment of the present invention is directed 25 to a peptide of approximately 30 amino acids, and its encoding nucleic acids, of the toporythmic protein Delta that contains a sequence which is cleaved by the metalloprotease-disintegrin Kuzbanian (Kuz), (herein termed "cleavage peptide") as well as derivatives (e.g., fragments) and 30 analogs thereof. For example, the Delta cleavage peptide consists of the sequence of about amino acid Cys₅₁₆ to about amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), of about

amino acid Cys₅₁₅ to about amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), of about amino acid Cys₅₂₃ to about amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), of about amino acid Cys₅₁₈ to about amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), and 5 the sequence of about amino acid Cys₅₆₄ to about amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). Such a peptide is believed to have the ability to modulate Kuz cleavage of Delta, and thus, Delta and Notch activation. In a specific embodiment, the Delta cleavage peptide is a portion of a mammalian Delta, preferably a human Delta.

10 The invention further relates to Delta cleavage peptides, and derivatives (including but not limited to fragments) and analogs of Delta cleavage peptides. Nucleic acids encoding Delta cleavage peptide derivatives and peptide analogs are also provided. In particular aspects, the 15 peptides, derivatives, or analogs are of mouse, chicken, frog, rat, pig, cow, dog, monkey, or human Delta cleavage peptides.

16 The production and use of derivatives and analogs related to Delta cleavage peptides are within the scope of 20 the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting one or more functional activities associated with wild-type Delta cleavage peptide. As one example, such derivatives or analogs which have the desired immunogenicity 25 or antigenicity can be used, for example, in immunoassays, for immunization, for inhibition of Delta activity, etc. Such molecules which retain, or alternatively inhibit, a desired Delta property, e.g., binding to kuz or other toporythmic proteins, can be used as inducers, or inhibitors, respectively, of such property and its physiological 30 correlates. Derivatives or analogs of a Delta cleavage peptide can be tested for the desired activity by procedures

known in the art, including but not limited to the assays described herein.

In particular, Delta cleavage peptide derivatives can be made by altering Delta cleavage peptide encoding sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a Delta cleavage peptide may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the encoding Delta cleavage peptide genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the Delta cleavage peptide derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a Delta protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The

negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment, fragments of Delta that comprise the cleavage peptide sequence are also provided. In 5 particular embodiments, the Delta fragments comprising the cleavage peptide are not greater than 35, 50, 75, 100, 150, or 200 amino acids in length. For example, a Delta fragment containing the cleavage peptide sequence comprises the cleavage peptide sequence and 35 contiguous amino-terminal amino acids. In another example, the fragment comprises the cleavage peptide sequence and 100 contiguous amino-terminal amino acids. In yet another example, the fragment comprises the cleavage peptide sequence and 50 contiguous carboxy-terminal amino acids. In yet another example, the fragment comprises the cleavage peptide sequence and 50 contiguous 10 amino-terminal amino acids and 50 contiguous carboxy-terminal amino acids. In yet another embodiment, oncatamers of Delta fragments containing at least the cleavage peptide sequence (e.g., two, three, or more copies of a portion of the Delta sequence consisting of at least the cleavage peptide 15 sequence) are also provided.

The Delta cleavage peptide derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the 25 cloned *Delta* gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification 30 if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of a Delta cleavage peptide, care should be taken to ensure that

the modified gene remains within the same translational reading frame as Delta, uninterrupted by translational stop signals.

Additionally, the Delta cleavage peptide-encoding 5 nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* 10 modification. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), etc. PCR primers containing sequence changes can be used in PCR to introduce such changes into the amplified fragments.

15 Manipulations of the Delta cleavage peptide sequence may also be made at the protein level. Included within the scope of the invention are Delta cleavage peptide fragments or other derivatives or analogs which are differentially modified during or after translation, e.g., by 20 glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, 25 including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives of Delta 30 cleavage peptide can be chemically synthesized. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the Delta sequence. Non-classical amino acids include but

are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, 5 designer amino acids such as β -methyl amino acids, α -methyl amino acids, and $N\alpha$ -methyl amino acids and amino acid analogs in general.

In a specific embodiment, the Delta cleavage peptide derivative is a chimeric, or fusion, peptide comprising a Delta cleavage peptide or fragment thereof 10 joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising a Delta cleavage peptide-coding sequence joined 15 in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by 20 methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. In a specific embodiment, a chimeric nucleic acid encoding a Delta cleavage peptide with a heterologous signal sequence is expressed such 25 that the chimeric protein is expressed extracellularly by the cell.

The invention is also directed to a derivative or analog of the cleavage peptide which is functionally active, i.e., capable of displaying one or more known functional activities associated with the "wild type" cleavage peptide. 30 Such functional activities include but are not limited to antigenicity [ability to bind (or compete with the cleavage peptide for binding) to an anti-Delta cleavage peptide

antibody], immunogenicity (ability to generate antibody which binds to the cleavage peptide), ability to bind (or compete with the cleavage peptide for binding) to Kuz. The invention is further directed to a fragment (and derivatives or analogs 5 thereof) of the Delta cleavage peptide which is able to bind to Kuz.

In another embodiment, the present invention is directed to a peptide comprising an amino-terminal fragment of a full length Delta protein, which fragment is cleaved from the full length Delta protein by two proteolytic 10 processing events, the cleavage of the signal peptide and the cleavage by Kuz, (herein termed "soluble Delta peptide") as well as derivatives and analogs thereof. For example, the soluble Delta peptide amino acid sequence begins at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₆ and amino 15 acid Phe₅₄₃ in human Delta (SEQ ID NO:10); begins at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); begins at amino acid Ser₂₄ and terminates between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), begins at amino acid Ser₂₂ 20 and terminates between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or begins at amino acid Ser₂₃ and terminates between amino acid Cys₅₆₄ and amino acid Ala₅₉₃, or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). Such a peptide 25 is believed to have the ability to bind Notch, and thus modulate Delta and Notch activation.

The invention further relates to soluble Delta peptides, and derivatives (including but not limited to fragments) and analogs of soluble Delta peptides. Nucleic acids encoding soluble Delta peptide derivatives and peptide analogs are also provided. In particular aspects, the 30 peptides, derivatives, or analogs are of mouse, chicken, frog, rat, pig, cow, dog, monkey, or human soluble Delta peptides.

The production and use of derivatives and analogs related to soluble Delta peptides are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting 5 one or more functional activities associated with wild-type soluble Delta peptide. As one example, such derivatives or analogs which have the desired immunogenicity or antigenicity can be used, for example, in immunoassays, for immunization, for promotion of Delta activity, etc. Such molecules which 10 retain, or alternatively inhibit, a desired Delta property, e.g., binding to Notch or other toporythmic proteins, can be used as inducers, or inhibitors, respectively, of such property and its physiological correlates. Derivatives or analogs of a soluble Delta peptide can be tested for the desired activity by procedures known in the art, including 15 but not limited to the assays described herein.

In particular, soluble Delta peptide derivatives can be made by altering soluble Delta peptide encoding sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the 20 degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a soluble Delta peptide may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the encoding soluble Delta peptide genes which are altered by 25 the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the soluble Delta peptide derivatives of the invention include, but are not limited to, those containing, as a primary amino 30 acid sequence, all or part of the amino acid sequence of a Delta protein including altered sequences in which functionally equivalent amino acid residues are substituted

for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, 5 resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids 10 include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

15 The soluble Delta peptide derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned *Delta* gene sequence can be modified by any of numerous 20 strategies known in the art (Maniatis, T., 1990, *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification 25 if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of a soluble Delta peptide, care should be taken to ensure that the modified gene remains within the same translational reading frame as *Delta*, uninterrupted by translational stop signals.

30 Additionally, the soluble Delta peptide-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or

termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art 5 can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, *J. Biol. Chem.* 253:6551), etc. PCR primers containing sequence changes can be used in PCR to introduce such changes into the amplified fragments.

10 Manipulations of the soluble Delta peptide sequence may also be made at the protein level. Included within the scope of the invention are soluble Delta peptide fragments or other derivatives or analogs which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by 15 known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, 20 chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc. In a specific embodiment, N- or C-terminal modifications are made, e.g., N-acetylation.

25 In addition, analogs and derivatives of soluble Delta peptide can be chemically synthesized. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the Delta sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid, hydroxyproline, 30 sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, designer amino acids such as β -methyl amino acids, α -methyl

amino acids, and $\text{N}\alpha$ -methyl amino acids and amino acid analogs in general.

In a specific embodiment, the soluble Delta peptide derivative is a chimeric, or fusion, peptide comprising a 5 soluble Delta peptide or fragment thereof joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising a soluble 10 Delta peptide-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods 15 commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. In a specific embodiment, a chimeric nucleic acid encoding a soluble Delta peptide with a heterologous signal sequence is expressed such that the 20 chimeric protein is expressed extracellularly by the cell.

The invention is also directed to a derivative or analog of the soluble peptide which is functionally active, i.e., capable of displaying one or more known functional activities associated with the "wild type" soluble peptide. 25 Such functional activities include but are not limited to antigenicity [ability to bind (or compete with the soluble peptide for binding) to an anti-soluble Delta peptide antibody], immunogenicity (ability to generate antibody which binds to the soluble peptide), ability to bind (or compete with the soluble peptide for binding) to Notch. The 30 invention is further directed to a fragment (and derivatives or analogs thereof) of the soluble Delta peptide which is able to bind to Notch.

5.1.2

**PROTEIN COMPLEXES OF DELTA AND KUZ
AND DELTA AND NOTCH**

The present invention is directed to a Delta:Kuz protein complex. The present invention is also directed to a 5 Dl^{EC} :Notch protein complex. *Delta*, *Kuz* and *Notch* have been cloned, see e.g., WO 92/19734, WO 97/01571 and WO 98/08933. Figure 2 depicts the amino acid sequences of several Notch homologs (SEQ ID NOS:1, 2, 3 and 4), including human Notch (SEQ ID NOS:1 and 2). Figure 3 depicts the amino acid sequences of several Delta homologs (SEQ ID NOS:5, 6, 7, 8 10 and 9) and the nucleic acid sequence encoding human Delta is depicted in Figure 4B (SEQ ID NO:13). The amino acid sequence (SEQ ID NO:12) of the human homolog of Kuz and its encoding nucleic acid sequence (SEQ ID NO:13) is depicted in Figures 5A and 5B, respectively. Dl^{EC} is the amino-terminal 15 fragment of full length Delta consisting of essentially the extracellular domain of wild-type Delta that is liberated when Kuz cleaves Delta. The Dl^{EC} fragment is soluble and begins at amino acid Ser₂₃ and terminates between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ 20 ID NO:9), begins at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), begins at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), begins at amino acid Ser₂₄ and terminates between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), 25 or begins at amino acid Ser₂₂ and terminates between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8).

In a preferred embodiment of the present invention, the Delta:Kuz complex or the Dl^{EC} :Notch complex is a complex 30 of human proteins. The invention is also directed to complexes of derivatives (including fragments) and analogs of Delta with Kuz, complexes of Delta with derivatives

(including fragments) and analogs of Kuz, and complexes of derivatives (including fragments) and analogs of Delta and Kuz (as used herein, fragment, derivative, homolog or analog of a Delta:Kuz complex includes complexes where one or both members of the complex are fragments, derivatives or analogs of the wild-type Delta or Kuz protein). The present invention is also directed to complexes of derivatives (including fragments) and analogs of Dl^{EC} with Notch, complexes of Dl^{EC} with derivatives (including fragments) and analogs of Notch, and complexes of derivatives (including fragments) and analogs of Dl^{EC} and Notch (as used herein, fragment, derivative, homolog or analog of a Dl^{EC}:Notch complex includes complexes where one or both members of the complex are fragments, derivatives or analogs of the wild-type Dl^{EC} or Notch protein). In a preferred embodiment, the Dl^{EC}:Notch complex in which one or both members of the complex is a fragment, derivative, homolog or analog of the wild type protein is a functionally-active Dl^{EC}:Notch complex. In particular aspects, the native proteins, or derivatives or analogs of Delta, Notch and/or Kuz are obtained from an animal, e.g., mouse, rat, pig, cow, dog, monkey, human, fly, frog. In another aspect, the native proteins are obtained from plants.

As used herein, a "functionally active Delta:Kuz complex" refers to that material displaying one or more known functional attributes of a complex of wild type Delta with wild type Kuz, including protein-protein binding, binding to a Delta-, a Kuz-, and/or a Delta:Kuz complex-specific antibody, or has the functional attribute(s) of Delta, Kuz, and/or a Delta:Kuz complex involved in cell fate and differentiation.

As used herein, a "functionally active Dl^{EC}:Notch complex" refers to that material displaying one or more known functional attributes of a complex of wild type Dl^{EC} with wild

type Notch, including protein-protein binding, binding to a Dl^{EC}-, a Notch-, and/or a Dl^{EC}:Notch complex-specific antibody, or has the functional attribute(s) of Dl^{EC}, Notch, and/or a Dl^{EC}:Notch complex involved in cell fate and differentiation.

5 The present invention is also directed to a method of screening a Delta:Kuz complex, particularly a complex of Delta with Kuz for the ability to alter a cell function, particularly those cell functions in which Delta and/or Kuz has been implicated, including, e.g., physiological processes such as cell fate determination and differentiation, binding to an anti-Delta:Kuz complex antibody, etc., and other activities as they are described in the art. The present invention is also directed to a method of screening a Dl^{EC}:Notch complex, particularly a complex of Dl^{EC} with Notch for the ability to alter a cell function, particularly those 10 cell functions in which Dl^{EC} and/or Notch has been implicated, including, e.g., physiological processes such as cell fate determination and differentiation, binding to an anti-Dl^{EC}:Notch complex antibody, etc., and other activities as they are described in the art.

15

20 The present invention is also directed to a method for screening a complex of a derivative, fragment, or analog of Delta and/or Kuz for the ability to alter a cell function such as differentiation. For example, such derivatives or analogs which have the desired immunogenicity or antigenicity can be used in immunoassays, for immunization, for inhibition 25 of Delta:Kuz complex activity, etc. Derivatives or analogs that retain, or alternatively lack or inhibit, a property of interest (e.g., participation in a Delta:Kuz complex) can be used as an inducer, or inhibitor, respectively, of such a property and its physiological correlate. The present invention is also directed to a method for screening a 30 complex of a derivative, fragment, or analog of Dl^{EC} and/or Notch for the ability to alter a cell function such as

differentiation. For example, such derivatives or analogs which have the desired immunogenicity or antigenicity can be used in immunoassays, for immunization, for inhibition of Dl^{EC}:Notch complex activity, etc. Derivatives or analogs that 5 retain, or alternatively lack or inhibit, a property of interest (e.g., participation in a Dl^{EC}:Notch complex) can be used as an inducer, or inhibitor, respectively, of such a property and its physiological correlate.

A specific embodiment of the present invention is directed to a Delta:Kuz complex of a fragment of Delta and/or a fragment of Kuz that can be bound by an anti-Delta antibody and/or bound by an anti-Kuz antibody, respectively, or bound by an antibody specific for a Delta:Kuz complex. Another specific embodiment of the present invention is directed to a Dl^{EC}:Notch complex of a fragment of Dl^{EC} and/or a fragment of 10 Notch that can be bound by an anti-Dl^{EC} antibody and/or bound by an anti-Notch antibody, respectively, or bound by an antibody specific for a Dl^{EC}:Notch complex. 15

Fragments and other derivatives or analogs of a Delta:Kuz complex or of a Dl^{EC}:Notch complex can be tested for 20 the desired activity by procedures known in the art, including but not limited to the assays described *infra*.

In specific embodiments, the present invention is directed to a Delta:Kuz complex or to a Dl^{EC}:Notch complex comprising a fragment of one or both members of the complex. 25 In a preferred embodiment, these fragments consist of, but are not exclusive to fragments of Kuz, identified as interacting with Delta in a modified yeast matrix mating assay or genetic screen. Fragments, or proteins comprising fragments, lacking a region of either member of the complex, are also provided. Nucleic acids encoding the foregoing are 30 provided in the present invention.

Nucleic acids encoding Delta, Notch and Kuz are known, and in addition can be obtained by any method known in

the art, e.g., by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of each sequence, and/or by cloning from a cDNA or genomic library using an oligonucleotide specific for each nucleotide sequence.

5 Homologs (e.g., nucleic acids encoding Delta, Notch and Kuz of species other than human) or other related sequences (e.g., paralogs) can be obtained by low, moderate or high stringency hybridization with all or a portion of the particular human sequence as a probe, using methods well known in the art for nucleic acid hybridization and cloning.

10 The encoded human Delta, Kuz and Notch proteins, which are depicted in Figures 4A, 5A and 2, respectively (SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NOS:1 and 2, respectively) either alone or in a complex, can be obtained by methods well known in the art for protein purification and recombinant 15 protein expression. For recombinant expression of one or more of the proteins, the nucleic acid containing all or a portion of the nucleotide sequence encoding the protein can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the 20 transcription and translation of the inserted protein coding sequence. The necessary transcriptional and translational signals can also be supplied by the native promoter of the Delta, Kuz and Notch genes, and/or their flanking regions.

25 A variety of host-vector systems may be utilized to express the protein coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed 30 with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized,

any one of a number of suitable transcription and translation elements may be used.

In a preferred embodiment, a Delta:Kuz complex is obtained by expressing the entire Delta coding sequence and 5 the entire Kuz coding sequence in the same cell, either under the control of the same promoter or two separate promoters. In yet another embodiment, a derivative, fragment or homolog of Delta and/or a derivative, fragment or homolog of Kuz are recombinantly expressed. Preferably the derivative, fragment or homolog of Delta and/or the Kuz protein forms a complex 10 with a binding partner identified by a binding assay, and more preferably forms a complex that binds to an anti-Delta:Kuz complex antibody. In another preferred embodiment, a SDelta:Notch complex is obtained by expressing the entire D^{1EC} coding sequence and the entire Notch coding sequence in 15 the same cell, either under the control of the same promoter or two separate promoters. In yet another embodiment, a derivative, fragment or homolog of D^{1EC} and/or a derivative, fragment or homolog of Notch are recombinantly expressed. Preferably the derivative, fragment or homolog of D^{1EC} and/or 20 the Notch protein forms a complex with a binding partner identified by a binding assay, and more preferably forms a complex that binds to an anti-D^{1EC}:Notch complex antibody.

Any method available in the art can be used for the insertion of DNA fragments into a vector to construct 25 expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinant techniques (genetic recombination). Expression of nucleic acid sequences encoding Delta, Kuz and Notch, or a 30 derivative, fragment or homolog thereof, may be regulated by a second nucleic acid sequence so that the gene or fragment thereof is expressed in a host transformed with the

recombinant DNA molecule(s). For example, expression of the proteins may be controlled by any promoter/enhancer known in the art. In a specific embodiment, the promoter is not native to the genes for Delta, Notch or Kuz. Promoters that 5 may be used include but are not limited to those described in Section 5.1.1.

In a specific embodiment, a vector is used that comprises a promoter operably linked to nucleic acid sequences encoding Delta, Notch and/or Kuz, or a fragment, derivative or homolog thereof, one or more origins of 10 replication, and optionally, one or more selectable markers (e.g., an antibiotic resistance gene). In a preferred embodiment, a vector is used that comprises a promoter operably linked to nucleic acid sequences encoding both Delta and Kuz, or both Dl^{EC} and Notch, one or more origins of 15 replication, and optionally, one or more selectable markers.

In another specific embodiment, an expression vector containing the coding sequence, or a portion thereof, of Delta and Kuz, or of Dl^{EC} and Notch, either together or separately, is made by subcloning the gene sequences into the 20 EcoRI restriction site of each of the three pGEX vectors (glutathione S-transferase expression vectors; Smith and Johnson, 1988, Gene 7:31-40). This allows for the expression of products in the correct reading frame.

Expression vectors containing the sequences of 25 interest can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene function, and (c) expression of the inserted sequences. In the first approach, Delta, Notch and Kuz sequences can be detected by nucleic acid hybridization to probes comprising sequences homologous and complementary to 30 the inserted sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker"

functions (e.g., resistance to antibiotics, occlusion body formation in baculovirus, etc.) caused by insertion of the sequences of interest in the vector. For example, if a Delta or Kuz gene, or portion thereof, is inserted within the 5 marker gene sequence of the vector, recombinants containing the Delta or Kuz fragment will be identified by the absence of the marker gene function (e.g., loss of beta-galactosidase activity). In the third approach, recombinant expression vectors can be identified by assaying for the Delta and Kuz expressed by the recombinant vector. Such assays can be 10 based, for example, on the physical or functional properties of the interacting species in *in vitro* assay systems, e.g., formation of a Delta:Kuz complex or binding to an anti-Delta, anti-Kuz, or anti-Delta:Kuz complex antibody.

Once recombinant Delta, Notch and Kuz molecules are 15 identified and the complexes or individual proteins isolated, several methods known in the art can be used to propagate them. Using a suitable host system and growth conditions, recombinant expression vectors can be propagated and amplified in quantity. As previously described, the 20 expression vectors or derivatives which can be used include, but are not limited to, human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus, yeast vectors; bacteriophage vectors such as lambda phage; and plasmid and cosmid vectors.

25 In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies or processes the expressed proteins in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus expression of the genetically-engineered Delta, Notch and/or Kuz may be 30 controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g.,

glycosylation, phosphorylation, etc.) of proteins. Appropriate cell lines or host systems can be chosen to ensure that the desired modification and processing of the foreign protein is achieved. For example, expression in a 5 bacterial system can be used to produce an unglycosylated core protein, while expression in mammalian cells ensures "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

10 In other specific embodiments, the Delta, Notch and/or Kuz protein or a fragment, homolog or derivative thereof, may be expressed as fusion or chimeric protein products comprising the protein, fragment, homolog, or derivative joined via a peptide bond to a heterologous protein sequence of a different protein. Such chimeric 15 products can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acids to each other by methods known in the art, in the proper coding frame, and expressing the chimeric products in a suitable host by methods commonly known in the art. Alternatively, such a 20 chimeric product can be made by protein synthetic techniques, e.g., by use of a peptide synthesizer. Chimeric genes comprising portions of Delta, Notch and/or Kuz fused to any heterologous protein-encoding sequences may be constructed. A specific embodiment relates to a chimeric protein 25 comprising a fragment of Delta, Notch and/or Kuz of at least six amino acids.

In a specific embodiment, fusion proteins are provided that contain the interacting domains of the Delta protein and Kuz, or the interacting domains of Dl^{EC} and Notch, and, optionally, a peptide linker between the two domains, 30 where such a linker promotes the interaction of the Delta and Kuz binding domains or promotes the interaction of the Dl^{EC} and Notch binding domains. These fusion proteins may be

particularly useful where the stability of the interaction is desirable (due to the formation of the complex as an intra-molecular reaction), for example, in production of antibodies specific to the Delta:Kuz complex or specific to the 5 D^{lec}:Notch complex.

In particular, Delta, Notch and/or Kuz derivatives can be made by altering their sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences that encode 10 substantially the same amino acid sequence as a Delta, Notch or Kuz gene or cDNA can be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the Delta, Notch or Kuz genes that are altered by the substitution of 15 different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the Delta, Notch or Kuz derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino 20 acid sequence of Delta, Notch or Kuz, including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity that acts as a 25 functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, 30 phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged

(basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, the 5 nucleic acids encoding proteins and proteins consisting of or comprising a fragment of Delta, Notch or Kuz consisting of at least 6 (continuous) amino acids of Delta, Notch or Kuz are provided. In other embodiments, the fragment consists of at least 10, 20, 30, 40, or 50 amino acids of Delta and Kuz or Dl^{EC} and Notch. In specific embodiments, such fragments are 10 not larger than 35, 100 or 200 amino acids. Derivatives or analogs of Delta, Notch and Kuz include, but are not limited, to molecules comprising regions that are substantially homologous to Delta, Notch or Kuz, in various embodiments, by at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% identity 15 over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art, or whose encoding nucleic acid is capable of hybridizing to a sequence encoding Delta, Notch or Kuz under stringent, 20 moderately stringent, or nonstringent conditions.

The Delta, Notch and Kuz derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned 25 Delta, Notch and Kuz gene sequences can be modified by any of numerous strategies known in the art (Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). The sequences can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic 30 modification if desired, isolated, and ligated in vitro. In the production of the gene encoding a derivative, homolog or analog of Delta, Notch or Kuz, care should be taken to ensure

that the modified gene retains the original translational reading frame, uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, the Delta-, Notch- and/or Kuz-
5 encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy pre-existing ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art
10 can be used, including but not limited to, chemical mutagenesis and *in vitro* site-directed mutagenesis (Hutchinson et al., 1978, *J. Biol. Chem.* 253:6551-6558), amplification with PCR primers containing a mutation, etc.

Once a recombinant cell expressing Delta, Notch
15 and/or Kuz, or fragment or derivative thereof, is identified, the individual gene product or complex can be isolated and analyzed. This is achieved by assays based on the physical and/or functional properties of the protein or complex, including, but not limited to, radioactive labeling of the
20 product followed by analysis by gel electrophoresis, immunoassay, cross-linking to marker-labeled product, etc.

The Delta:Kuz or Dl^{EC}:Notch complexes may be isolated and purified by standard methods known in the art (either from natural sources or recombinant host cells
25 expressing the complexes or proteins), including but not restricted to column chromatography (e.g., ion exchange, affinity, gel exclusion, reversed-phase high pressure, fast protein liquid, etc.), differential centrifugation, differential solubility, or by any other standard technique used for the purification of proteins. Functional properties
30 may be evaluated using any suitable assay known in the art.

Alternatively, once Delta or its derivative, or Kuz or its derivative, or Notch or its derivative, is identified,

the amino acid sequence of the protein can be deduced from the nucleic acid sequence of the chimeric gene from which it was encoded. As a result, the protein or its derivative can be synthesized by standard chemical methods known in the art 5 (e.g., Hunkapiller et al., 1984, *Nature* 310: 105-111).

In a specific embodiment of the present invention, such Delta:Kuz complexes, whether produced by recombinant DNA techniques, chemical synthesis methods, or by purification from native sources include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequences substantially as depicted in Figures 10 3 and 5A-5B (SEQ ID NOS:5, 6, 7, 8 and 9 and SEQ ID NO:12, respectively), as well as fragments and other analogs and derivatives thereof, including proteins homologous thereto. In another specific embodiment of the present invention, such 15 D^{lec}:Notch complexes, whether produced by recombinant DNA techniques, chemical synthesis methods, or by purification from native sources include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequences substantially as depicted in Figures 20 2 and 3 (SEQ ID NOS:5, 6, 7, 8 and 9 and SEQ ID NOS:1, 2, 3, and 4, respectively), as well as fragments and other analogs and derivatives thereof, including proteins homologous thereto.

Manipulations of Delta, Notch and/or Kuz sequences 25 may be made at the protein level. Included within the scope of one embodiment of the invention is a complex of a Delta fragment or a Kuz fragment and Delta or Kuz fragments, derivatives and analogs that are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by 30 known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried

out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄, acetylation, formylation, oxidation, reduction, metabolic synthesis in the 5 presence of tunicamycin, etc.

In specific embodiments, the Delta, Notch and/or Kuz amino acid sequences are modified to include a fluorescent label. In another specific embodiment, Delta, Notch and/or Kuz are modified to have a heterofunctional reagent; such heterofunctional reagents can be used to 10 crosslink the members of the complex.

In addition, complexes of analogs and derivatives of Delta and/or Kuz, or D^{1_{EC}} and/or Notch, can be chemically synthesized. For example, a peptide corresponding to a portion of Delta and/or Kuz, which comprises the desired 15 domain or mediates the desired activity *in vitro* (e.g., Delta:Kuz complex formation) can be synthesized by use of a peptide synthesizer. Furthermore, if desired, non-classical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the Delta and/or Kuz. 20 Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid (4-Abu), 2-aminobutyric acid (2- Abu), 6-amino hexanoic acid (Ahx), 2-amino isobutyric acid (2-Aib), 3-amino propionic acid, ornithine, norleucine, norvaline, 25 hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β -methyl amino acids, α -methyl amino acids, $\text{N}\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) 30 or L (levorotary).

In cases where natural products are suspected of being mutant or are isolated from new species, the amino acid

sequence of Delta, Notch or Kuz isolated from the natural source, as well as those expressed *in vitro*, or from synthesized expression vectors *in vivo* or *in vitro*, can be determined from analysis of the DNA sequence, or 5 alternatively, by direct sequencing of the isolated protein. Such analysis can be performed by manual sequencing or through use of an automated amino acid sequenator.

The Delta:Kuz or D^{lec}:Notch complexes can also be analyzed by hydrophilicity analysis (Hopp and Woods, 1981, 10 Proc. Natl. Acad. Sci. USA 78:3824-3828). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the proteins, and help predict their orientation in designing substrates for experimental manipulation, such as in binding experiments, antibody synthesis, etc. Secondary structural analysis can also be 15 done to identify regions of Delta, Notch and/or Kuz, or their derivatives, that assume specific structures (Chou and Fasman, 1974, Biochemistry 13:222-23). Manipulation, translation, secondary structure prediction, hydrophilicity and hydrophobicity profile predictions, open reading frame 20 prediction and plotting, and determination of sequence homologies, etc., can be accomplished using computer software programs available in the art.

Other methods of structural analysis including but not limited to X-ray crystallography (Engstrom, 1974 Biochem. 25 Exp. Biol. 11:7-13), mass spectroscopy and gas chromatography (Methods in Protein Science, J. Wiley and Sons, New York, 1997), and computer modeling (Fletterick and Zoller, eds., 1986, Computer Graphics and Molecular Modeling, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, New York) can also be 30 employed.

5.2 ANTIBODIES

According to one embodiment of the present invention, a Delta cleavage peptide, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which recognize such an immunogen.

According to another embodiment of the present invention, a soluble Delta peptide, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which recognize such an immunogen. Such antibodies include but are not limited to polyclonal, 10 monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to human Delta cleavage peptide are produced. In another specific embodiment, antibodies to human soluble Delta peptide are produced.

According to another embodiment of the present invention, the Delta:Kuz complex or a fragment, derivative or homolog thereof, or the Dl^{EC}:Notch complex or a fragment, derivative or homolog thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such 20 immunogen. Such antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to a complex of human Delta and human Kuz are produced. In another specific embodiment, antibodies to a complex of human Dl^{EC} and human Notch are produced. In 25 another embodiment, a complex formed from a fragment of Delta and a fragment of Kuz, which fragments contain the protein domain that interacts with the other member of the complex, are used as an immunogen for antibody production.

Various procedures known in the art may be used for 30 the production of polyclonal antibodies to a Delta cleavage peptide or derivative or analog, or to a soluble Delta peptide or derivative or analog, or to a protein complex of

the present invention. For the production of antibody, various host animals can be immunized by injection with the native Delta cleavage peptide, or D1^{EC} or Notch or Kuz, or a synthetic version, or derivative (e.g., fragment) thereof, 5 including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances 10 such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille 15 Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward, for example, a Delta cleavage peptide sequence or 20 analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B- 25 cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, 30 monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies"

(Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for, e.g., 5 Delta cleavage peptide together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 10 4,946,778) can be adapted to produce, for example, Delta cleavage peptide-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments 15 with the desired specificity for Delta proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the 20 F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. 25

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, e.g. ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize, for example, a Delta cleavage peptide, one may assay generated hybridomas 30 for a product which binds to a Delta cleavage peptide. For selection of an antibody immunospecific to human Delta cleavage peptide, one can select on the basis of positive

binding to human Delta cleavage peptide and a lack of binding to *Drosophila* Delta cleavage peptide.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of 5 the protein sequences of the invention, e.g., for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

In another embodiment of the invention (see *infra*), anti-Delta cleavage peptide antibodies specific for the Delta 10 cleavage peptide and fragments thereof containing the binding domain are Therapeutics. In yet another embodiment of the invention, an anti-Delta:Kuz complex antibody or a fragment thereof containing the binding domain, is a Therapeutic. In yet another embodiment of the invention, an anti-soluble Delta peptide antibody or a fragment thereof containing the 15 binding domain, is a Therapeutic.

5.3 DETECTION OF THE ACTIVE FORM OF DELTA

The present invention is directed to methods for detecting or measuring Delta activation by observing or 20 measuring Delta cleavage products that are indicative of Delta activation. In one aspect of this embodiment of the invention, the method for detecting or measuring Delta activation in a cell comprises detecting or measuring the expression of one or more Delta cleavage products selected 25 from the group consisting of D₁^{EC} and D₁TM. In yet another aspect, the method comprises detecting or measuring an amino-terminal fragment of full-length Delta beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9), beginning at amino acid Ser₂₂ and terminating between amino 30 acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID

NO:6), beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), or beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* 5 Delta (SEQ ID NO:8). In yet another aspect, the method comprises detecting or measuring under reducing conditions, a soluble Delta fragment of approximately 67 kilodaltons.

In another embodiment, the present invention is also directed to methods for identifying a molecule that modulates Delta activation by detecting or measuring a change 10 in the amount or pattern of Delta cleavage products. In one aspect of this embodiment of the invention, the method for identifying a modulator of Notch activation comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or 15 more Notch cleavage products selected from the group consisting of Dl^{EC} and DlTM, in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Delta 20 activity.

In an alternative aspect, the method for identifying a modulator of Delta activation comprises contacting a candidate modulator molecule with a full length Delta in the presence of Kuz and optionally a composition comprising cellular proteins, under conditions conducive to 25 cleavage of the full-length Delta by Kuz and optionally one or more components of the composition and detecting or measuring the amount of Delta cleavage products Dl^{EC} and DlTM that result, in which a difference in the presence or amount of said Notch cleavage products compared to a full-length 30 Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

Any method known in the art for detecting or measuring the expression of Delta cleavage products indicative of Delta activation can be used. For example, and not by way of limitation, one such method of detection of the 5 active form of Delta by detecting one or more Delta cleavage products selected from the group consisting of D1^{EC} and D1TM, or by detecting an amino-terminal fragment of full-length Delta beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9), beginning at amino acid Ser₂₂ and 10 terminating between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in 15 chick Delta (SEQ ID NO:7), or beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8). In yet another aspect, the method comprises detecting or measuring under reducing conditions, a soluble Delta fragment of approximately 67 20 kilodaltons.

Detection of such cleavage products can be done, e.g., by immunoprecipitating the cleavage products with an anti-Delta antibody or binding to anti-Delta antibody on an immunoaffinity column or immobilized on a plate or in a well, or visualizing the fragments by Western blotting. In a 25 specific embodiment, the cleavage products can be labelled by general cell surface labeling, or, alternatively, by pulse labeling the cells by incubation in culture medium containing a radioactive label, or, alternatively, it can be anti-Delta antibody (or antibody binding partner) that is labeled rather 30 than the Delta cleavage products.

Another method to detect the active form of Delta is to use a Delta ligand or binding fragment thereof, such as

Notch, to bind to Delta (e.g., when the ligand is labeled), or to recover Delta by coimmunoprecipitating with the appropriate anti-Delta ligand antibody to coimmunoprecipitate Delta cleavage products, etc.

5 Similar procedures to those described *supra* can be used to make antibodies to domains of other proteins (particularly toporythmic proteins) that bind or otherwise interact with Delta (e.g., binding fragments of Notch).

10 The cell in which Delta activation is detected or measured can be any cell, e.g., one that endogenously or recombinantly expresses Delta. The cell can be vertebrate, insect (e.g., *Drosophila*), *C. elegans*, mammalian, bovine, murine, rat, avian, fish, primate, human, etc. The Delta which is expressed can be vertebrate, insect, *C. elegans*, mammalian, bovine, murine, rat, avian, fish, primate, human, etc. 15 The cell can be a cell of primary tissue, a cell line, or of an animal containing and expressing a Delta transgene. For example, the transgenic animal can be a *Drosophila* (e.g., *melanogaster*) or a *C. elegans*. In a preferred embodiment, the transgene encodes a human Delta. Transgenic animals can 20 be made by standard methods well known in the art (e.g., by use of P element transposons as a vector in *Drosophila*).

5.4 METHODS OF IDENTIFYING MODULATORS OF DELTA ACTIVATION

25 In one embodiment of the invention, methods are provided for the identification of modulators, e.g., inhibitors, antagonists, or agonists, of Delta activation by detecting the ability of the modulators to effect cleavage of full length Delta. In one aspect of this embodiment of the invention, the method for identifying a modulator of Delta 30 activation comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more Delta cleavage products selected

from the group consisting of D1^{EC} and D1TM, in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates 5 Delta activity. In yet another aspect, the method comprises providing a cell with a candidate modulator molecule and detecting or measuring the amount of the expression by the cell of an amino-terminal fragment of full-length Delta beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta 10 (SEQ ID NO:9), beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), beginning at amino acid Ser₂₄ and terminating 15 between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), or beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); in which a difference in the presence or amount of said fragment compared to a Delta cell 20 not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

In yet another aspect, the method comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of a soluble Delta fragment of approximately 67 kilodaltons, in 25 which a difference in the presence or amount of said soluble fragment compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

In yet another aspect of this embodiment of the 30 invention, the method for identifying a modulator of Delta activation comprises contacting a candidate modulator molecule with a full length Delta in the presence of Kuz and

optionally a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of the composition, and detecting or measuring the amount of Delta cleavage

5 products $D\delta^{\text{sc}}$ and/or $D\delta^{\text{TM}}$ that result, in which a difference in the presence or amount of said Delta cleavage product(s) compared to a full-length Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Delta activity. In

10 another aspect, the method for identifying a modulator of Delta activation comprises contacting a candidate modulator molecule with a full length Delta in the presence of Kuz and optionally a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of the composition,

15 and detecting or measuring an amino-terminal fragment of full-length Delta beginning at amino acid Ser_{23} and terminating between amino acid Cys_{564} and amino acid Ala_{593} or Gln_{594} in *Drosophila* Delta (SEQ ID NO:9), beginning at amino acid Ser_{22} and terminating between amino acid Cys_{516} and amino

20 acid Phe_{543} in human Delta (SEQ ID NO:10), beginning at amino acid Ser_{22} and terminating between amino acid Cys_{515} and amino acid Phe_{543} in mouse Delta (SEQ ID NO:6), beginning at amino acid Ser_{24} and terminating between amino acid Cys_{523} and amino acid Phe_{551} in chick Delta (SEQ ID NO:7), or beginning at

25 amino acid Ser_{22} and terminating between amino acid Cys_{518} and amino acid Phe_{544} in *Xenopus* Delta (SEQ ID NO:8), in which a difference in the presence or amount of said fragment compared to a full-length Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

30 In yet another aspect, the method for identifying a modulator of Delta activation comprises contacting a candidate modulator molecule with a full length Delta in the

presence of Kuz and optionally a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of the composition and detecting or measuring the 5 amount of a soluble Delta fragment of approximately 67 kilodaltons, in which a difference in the presence or amount of said soluble fragment compared to a full-length Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

10 In a specific aspect of the embodiment using a composition comprising cellular proteins, the composition comprising cellular proteins is a cell lysate made from cells which recombinantly express Delta. In another specific aspect of this embodiment, the composition comprising 15 cellular proteins is a cell lysate made from cells which endogenously express Delta.

Detection or measurement of Delta cleavage products can be carried out by methods well known in the art and/or those methods disclosed in Section 5.1, *supra*.

20 The cells used in the methods of this embodiment can either endogenously or recombinantly express Delta. Examples of the cell types and Delta protein that can be expressed are described in Section 5.1. Recombinant Delta expression is carried out by introducing Delta encoding 25 nucleic acids into expression vectors and subsequently introducing the vectors into a cell to express Delta or simply introducing Delta encoding nucleic acids into a cell for expression. Nucleic acids encoding vertebrate and non-vertebrate Delta have been cloned and sequenced and their expression is well known in the art. See, for example, 30 International Publication WO 97/01571, which is incorporated by reference in their entirety herein. Expression can be from expression vectors or intrachromosomal.

Any method known to those of skill in the art for the insertion of Delta-DNA into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational 5 control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a Delta protein may be regulated by a second nucleic acid sequence so that 10 the Delta protein is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a Delta protein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control Delta gene expression include, but are not limited to, those described in Section 5.1.

15 In the methods of the invention in which full-length Delta is incubated with compositions comprising cellular proteins (e.g., cell lysates or cell fractions) in the presence of candidate cleavage (and thus Delta activation) modulators the expression of Delta should be such 20 that full length Delta is expressed and proteolytic cleavage of Delta is kept to a minimum such that Delta cleavage products are easily detected over any background proteolysis. There are several methods known in the art to keep 25 proteolysis to a minimum. For example, one manner to keep Delta cleavage to a minimum is to express Delta in cells concurrently with Brefeldin A treatment. Another manner is to express Delta in cells which do not contain Kuz or to express Delta in an *in vitro* transcription-translation system in the presence of a protease inhibitor such as phenylmethylsulfonylfluoride (PMSF).

30

5.5 METHODS OF IDENTIFYING MODULATORS OF KUZ ACTIVATION

In one embodiment of the invention, methods are provided for the identification of modulators, e.g., 5 inhibitors, antagonists, or agonists, of Kuz function by detecting the ability of the modulators to effect cleavage of full length Delta. In one aspect of this embodiment of the invention, the method for identifying a modulator of Kuz function comprises providing a Delta expressing cell with a candidate modulator molecule and detecting or measuring the 10 expression by the cell of one or more Delta cleavage products selected from the group consisting of D1^{EC} and D1TM, in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates 15 Kuz function. In yet another aspect, the method comprises providing a Delta expressing cell with a candidate modulator molecule and detecting or measuring the amount of the expression by the cell of an amino-terminal fragment of full-length Delta beginning at amino acid Ser₂₃ and terminating 20 between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9), beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ 25 in mouse Delta (SEQ ID NO:6), beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), or beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ 30 in *Xenopus* Delta (SEQ ID NO:8); in which a difference in the presence or amount of said fragment compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Kuz function.

In yet another aspect, the method comprises providing a Delta expressing cell with a candidate modulator molecule and detecting or measuring the expression by the cell of a soluble Delta fragment of approximately 67 kilodaltons, in which a difference in the presence or amount of said soluble fragment compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Kuz function.

In yet another aspect of this embodiment of the invention, the method for identifying a modulator of Kuz function comprises contacting a candidate modulator molecule with a full length Delta in the presence of Kuz and optionally a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of the composition, and detecting or measuring the amount of Delta cleavage products $D1^{EC}$ and/or $D1^{TM}$ that result, in which a difference in the presence or amount of said Delta cleavage product(s) compared to a full-length Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Kuz activity. In another aspect, the method for identifying a modulator of Kuz function comprises contacting a candidate modulator molecule with a full length Delta in the presence of Kuz and optionally a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of the composition, and detecting or measuring an amino-terminal fragment of full-length Delta beginning at amino acid Ser_{22} and terminating between amino acid Cys_{564} and amino acid Ala_{593} or Gln_{594} in *Drosophila* Delta (SEQ ID NO:9), beginning at amino acid Ser_{22} and terminating between amino acid Cys_{516} and amino acid Phe_{543} in human Delta (SEQ ID NO:10), beginning at amino acid Ser_{22} and terminating between amino acid Cys_{515} and amino

acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), or beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and 5 amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), in which a difference in the presence or amount of said fragment compared to a full-length Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Kuz function.

10 In yet another aspect, the method for identifying a modulator of Kuz function comprises contacting a candidate modulator molecule with a full length Delta in the presence of Kuz and optionally a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of 15 the composition and detecting or measuring the amount of a soluble Delta fragment of approximately 67 kilodaltons, in which a difference in the presence or amount of said soluble fragment compared to a full-length Delta in presence of said composition not contacted with the candidate molecule 20 indicates that the molecule modulates Kuz function.

In a specific aspect of the embodiment using a composition comprising cellular proteins, the composition comprising cellular proteins is a cell lysate made from cells which recombinantly express Kuz. In another specific aspect 25 of this embodiment, the composition comprising cellular proteins is a cell lysate made from cells which endogenously express Kuz.

Detection or measurement of Delta cleavage products can be carried out by methods well known in the art and/or those methods disclosed in Section 5.1, *supra*.

30 The cells used in the methods of this embodiment can either endogenously or recombinantly express Kuz. Examples of the cell types and Kuz protein that can be

expressed are described in Section 5.1. Recombinant Kuz expression is carried out by introducing Kuz encoding nucleic acids into expression vectors and subsequently introducing the vectors into a cell to express Kuz or simply introducing 5 Kuz encoding nucleic acids into a cell for expression.

Nucleic acids encoding vertebrate and non-vertebrate Kuz have been cloned and sequenced and their expression is well known in the art. See, for example, International Publication WO 98/08933, which is incorporated by reference in its entirety 10 herein. Expression can be from expression vectors or intrachromosomal.

Any method known to those of skill in the art for the insertion of Kuz-DNA into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational 15 control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a Delta or Kuz protein may be regulated by a second nucleic acid sequence so 20 that the Kuz protein is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a Kuz protein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control Delta gene expression include, but are not limited 25 to, those described in Section 5.1.

In the methods of the invention in which full-length Delta and Kuz is incubated with compositions comprising cellular proteins (e.g., cell lysates or cell fractions) in the presence of candidate cleavage (and thus Delta activation) modulators the expression of Delta should 30 be such that full length Delta is expressed and proteolytic cleavage of Delta is kept to a minimum such that Delta cleavage products are easily detected over any background

proteolysis. There are several methods known in the art to keep proteolysis to a minimum. For example, one manner to keep Delta cleavage to a minimum is to express Delta in cells concurrently with Brefeldin A treatment. Another manner is 5 to express Kuz in cells which do not contain Delta or to express Kuz in an *in vitro* transcription-translation system in the presence of a protease inhibitor such as phenylmethylsulfonylfluoride (PMSF).

10 5.6 **METHODS OF IDENTIFYING MODULATORS OF DELTA:KUZ
COMPLEX OR D1^{EC}:NOTCH COMPLEX ACTIVITY**

Delta:Kuz or D1^{EC}:Notch complexes, and derivatives, fragments and analogs thereof, nucleic acids encoding Delta, Notch and Kuz as well as derivatives, fragments and analogs of the nucleic acids, can be used to screen for compounds 15 that bind to, or modulate the function of a Delta:Kuz complex or a D1^{EC}:Notch complex, complex member encoding nucleic acids, complex member proteins, and derivatives of the foregoing, and thus, have potential use as agonists or antagonists of Delta:Kuz or D1^{EC}:Notch complex activity or 20 formation. The present invention is thus directed to assays for detecting molecules that specifically bind to, or modulate the function of, Delta, Notch and Kuz nucleic acids, proteins or derivatives of the nucleic acids and proteins. For example, recombinant cells expressing both Delta and Kuz nucleic acids can be used to recombinantly produce the 25 complexes or proteins in these assays, to screen for molecules that bind to, or interfere with, or promote Delta:Kuz complex formation or activity. In preferred embodiments, polypeptide analogs that have superior stabilities but retain the ability to form a Delta:Kuz or 30 D1^{EC}:Notch complex (e.g., Delta and Kuz or D1^{EC} and Notch modified to be resistant to proteolytic degradation in the binding assay buffers, or to be resistant to oxidative

degradation), are used to screen for modulators of Delta activity or Kuz activity or Delta:Kuz complex activity or formation, or are used to screen for modulators of Dl^{EC} activity or Notch activity or Dl^{EC}:Notch complex activity or formation. Such resistant molecules can be generated, e.g., by substitution of amino acids at proteolytic cleavage sites, the use of chemically derivatized amino acids at proteolytic susceptible sites, and the replacement of amino acid residues subject to oxidation, i.e. methionine and cysteine.

10 A molecule (e.g., a putative binding partner or modulator of Delta:Kuz or Dl^{EC}:Notch complex activity or formation) is contacted with the Delta:Kuz or Dl^{EC}:Notch complex, or fragment thereof, respectively, under conditions conducive to binding or modulation, and then a molecule that specifically bind to or modulate Delta:Kuz or Dl^{EC}:Notch 15 complex activity or formation is identified. Similar methods can be used to screen for molecules that bind to or modulate the function of Delta:Kuz or Dl^{EC}:Notch complex encoding nucleic acids or derivatives thereof.

20 A particular aspect of the present invention relates to identifying molecules that inhibit or promote formation or degradation of a Delta:Kuz or Dl^{EC}:Notch complex, e.g., using the method described for screening inhibitors using the modified yeast matrix mating test described in International Patent Publication WO 97/47763 entitled 25 "Identification and Comparison of Protein-Protein Interactions that Occur in Populations and Identification of Inhibitors of These Interactions", which is incorporated by reference herein in its entirety.

20 In one embodiment of the invention, a molecule that modulates activity of Delta or Kuz, or a complex of Delta and Kuz, is identified by contacting one or more candidate molecules with Delta in the presence of Kuz; and measuring the amount of complex that forms between Delta and Kuz;

wherein an increase or decrease in the amount of complex that forms relative to the amount that forms in the absence of the candidate molecule(s) indicates that the molecule(s) modulates the activity of Delta or Kuz or said complex of 5 Delta and Kuz. In preferred embodiments, a modulator is identified by administering a candidate molecule to a transgenic non-human animal expressing both Delta and Kuz from promoters that are not the native Delta or the native Kuz promoters, more preferably where the candidate molecule is also recombinantly expressed in the transgenic non-human 10 animal. Alternatively, the method for identifying such a modulator can be carried out *in vitro*, preferably with purified Delta, purified Kuz, and a purified candidate molecule.

In another embodiment of the invention, a molecule 15 that modulates activity of Dl^{EC} or Notch, or a complex of Dl^{EC} and Notch, is identified by contacting one or more candidate molecules with Dl^{EC} in the presence of Notch; and measuring the amount of complex that forms between Dl^{EC} and Notch; wherein an increase or decrease in the amount of complex that 20 forms relative to the amount that forms in the absence of the candidate molecule(s) indicates that the molecule(s) modulates the activity of Dl^{EC} or Notch or said complex of Dl^{EC} and Notch. In preferred embodiments, a modulator is identified by administering a candidate molecule to a transgenic non-human animal expressing both Dl^{EC} and Notch 25 from promoters that are not the native Dl^{EC} or the native Notch promoters, more preferably where the candidate molecule is also recombinantly expressed in the transgenic non-human animal. Alternatively, the method for identifying such a modulator can be carried out *in vitro*, preferably with 30 purified Dl^{EC} , purified Notch, and a purified candidate molecule.

Methods that can be used to carry out the foregoing are commonly known in the art. Agents/molecules to be screened can be provided as mixtures of a limited number of specified compounds, or as compound libraries, peptide 5 libraries and the like as described in Section 5.7, *infra*. Agents/molecules to be screened may also include all forms of antisera, antisense nucleic acids, etc., that can modulate complex activity or formation.

10

5.7 CANDIDATE MOLECULES

Any molecule known in the art can be tested for its ability to modulate Delta activation or Kuz function as measured by the expression of one or more of the Delta cleavage products disclosed herein. Furthermore, any molecule known in the art can be tested for its ability to 15 modulate Delta:Kuz complex function, or for its ability to modulate Dl^{EC}:Notch complex function. For identifying a molecule that modulates Delta activation or Kuz function, candidate molecules can be directly provided to a cell expressing Delta or Kuz or, in the case of candidate 20 proteins, can be provided by providing their encoding nucleic acids under conditions in which the nucleic acids are recombinantly expressed to produce the candidate proteins within the Delta or Kuz expressing cell. In an embodiment of the invention directed to the assay using full-length Delta and a composition comprising cellular proteins, candidate 25 molecules can also be added to a composition comprising cellular proteins (whole cell lysates, membrane fraction, etc.), preferably derived from cells endogenously or recombinantly expressing Delta.

This embodiment of the invention is well suited to 30 screen chemical libraries for molecules which modulate, e.g., inhibit, antagonize, or agonize, Delta activation or Kuz function or complex function. The chemical libraries can be

peptide libraries, peptidomimetic libraries, other non-peptide synthetic organic libraries, etc.

Exemplary libraries are commercially available from several sources (ArQule, Tripos/PanLabs, ChemDesign, 5 Pharmacopoeia). In some cases, these chemical libraries are generated using combinatorial strategies that encode the identity of each member of the library on a substrate to which the member compound is attached, thus allowing direct and immediate identification of a molecule that is an effective modulator. Thus, in many combinatorial approaches, 10 the position on a plate of a compound specifies that compound's composition. Also, in one example, a single plate position may have from 1-20 chemicals that can be screened by administration to a well containing the interactions of interest. Thus, if modulation is detected, smaller and 15 smaller pools of interacting pairs can be assayed for the modulation activity. By such methods, many candidate molecules can be screened.

Many diversity libraries suitable for use are known in the art and can be used to provide compounds to be tested 20 according to the present invention. Alternatively, libraries can be constructed using standard methods. Chemical (synthetic) libraries, recombinant expression libraries, or polysome-based libraries are exemplary types of libraries that can be used.

The libraries can be constrained or semirigid 25 (having some degree of structural rigidity), or linear or nonconstrained. The library can be a cDNA or genomic expression library, random peptide expression library or a chemically synthesized random peptide library, or non-peptide library. Expression libraries are introduced into the cells 30 in which the assay occurs, where the nucleic acids of the library are expressed to produce their encoded proteins.

In one embodiment, peptide libraries that can be used in the present invention may be libraries that are chemically synthesized *in vitro*. Examples of such libraries are given in Houghten et al., 1991, *Nature* 354:84-86, which 5 describes mixtures of free hexapeptides in which the first and second residues in each peptide were individually and specifically defined; Lam et al., 1991, *Nature* 354:82-84, which describes a "one bead, one peptide" approach in which a solid phase split synthesis scheme produced a library of peptides in which each bead in the collection had immobilized 10 thereon a single, random sequence of amino acid residues; Medynski, 1994, *Bio/Technology* 12:709-710, which describes split synthesis and T-bag synthesis methods; and Gallop et al., 1994, *J. Medicinal Chemistry* 37(9):1233-1251. Simply by way of other examples, a combinatorial library may be 15 prepared for use, according to the methods of Ohlmeyer et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:10922-10926; Erb et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:11422-11426; Houghten et al., 1992, *Biotechniques* 13:412; Jayawickreme et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:1614-1618; or Salmon 20 et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:11708-11712. PCT Publication No. WO 93/20242 and Brenner and Lerner, 1992, *Proc. Natl. Acad. Sci. USA* 89:5381-5383 describe "encoded combinatorial chemical libraries," that contain oligonucleotide identifiers for each chemical polymer library member. 25

Further, more general, structurally constrained, organic diversity (e.g., nonpeptide) libraries, can also be used. By way of example, a benzodiazepine library (see e.g., Bunin et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:4708-4712) may be used.

30 Conformationally constrained libraries that can be used include but are not limited to those containing invariant cysteine residues which, in an oxidizing

environment, cross-link by disulfide bonds to form cystines, modified peptides (e.g., incorporating fluorine, metals, isotopic labels, are phosphorylated, etc.), peptides containing one or more non-naturally occurring amino acids, 5 non-peptide structures, and peptides containing a significant fraction of γ -carboxyglutamic acid.

Libraries of non-peptides, e.g., peptide derivatives (for example, that contain one or more non-naturally occurring amino acids) can also be used. One 10 example of these are peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371). Peptoids are polymers of non-natural amino acids that have naturally occurring side chains attached not to the alpha carbon but to the backbone amino nitrogen. Since peptoids are not easily degraded by human digestive enzymes, they are advantageously 15 more easily adaptable to drug use. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al., 1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

20 The members of the peptide libraries that can be screened according to the invention are not limited to containing the 20 naturally occurring amino acids. In particular, chemically synthesized libraries and polysome based libraries allow the use of amino acids in addition to 25 the 20 naturally occurring amino acids (by their inclusion in the precursor pool of amino acids used in library production). In specific embodiments, the library members contain one or more non-natural or non-classical amino acids or cyclic peptides. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, 30 α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid; γ -Abu, ϵ -Ahx, 6-amino hexanoic acid; Aib, 2-amino isobutyric acid; 3-amino propionic acid; ornithine;

norleucine; norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, designer amino acids such as β -methyl amino acids, α -methyl amino acids, $\text{N}\alpha$ -methyl amino acids, fluoro-amino acids and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

Further, toporythmic proteins, derivatives and fragments thereof, can be tested for the ability to modulate Delta activation. Toporythmic proteins, and more generally, members of the "Notch cascade" or the "Notch group" of genes, include Notch, Delta, Serrate, Kuz, and other members of the Delta/Serrate family, which are identified by genetic (as detected phenotypically, e.g., in *Drosophila*) or molecular interaction (e.g., binding *in vitro*). See, International Publications WO 92/19734, WO 97/18822, WO 96/27610, and WO 97/01571 and references therein, for examples of vertebrate and non-vertebrate members of the Notch family of genes.

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, e.g., the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, *Adv. Exp. Med. Biol.* 251:215-218; Scott and Smith, 1990, *Science* 249:386-390; Fowlkes et al., 1992, *BioTechniques* 13:422-427; Oldenburg et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:5393-5397; Yu et al., 1994, *Cell* 76:933-945; Staudt et al., 1988, *Science* 241:577-580; Bock et al., 1992, *Nature* 355:564-566; Tuerk et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:6988-6992; Ellington et al., 1992, *Nature* 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, *Science* 263:671-673; and International Patent Publication No. WO 94/18318.

In a specific embodiment, screening can be carried out by contacting the library members with Delta or Kuz or a

protein complex or the present invention (or encoding nucleic acid or derivative) immobilized on a solid phase, and harvesting those library members that bind to the protein or complex (or encoding nucleic acid or derivative). Examples 5 of such screening methods, termed "panning" techniques, are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; International Patent Publication No. WO 94/18318; and in references cited hereinabove.

10 In a specific embodiment, fragments and/or analogs of Delta or Kuz, especially peptidomimetics, are screened for activity as competitive or non-competitive inhibitors of Delta:Kuz complex formation, which thereby inhibit Delta:Kuz complex activity or formation.

Methods for screening may involve labeling the 15 proteins or complex proteins of the present invention with radioligands (e.g., ^{125}I or ^3H), magnetic ligands (e.g., paramagnetic beads covalently attached to photobiotin acetate), fluorescent ligands (e.g., fluorescein or rhodamine), or enzyme ligands (e.g., luciferase or beta-20 galactosidase). The reactants that bind in solution can then be isolated by one of many techniques known in the art, including but not restricted to, co-immunoprecipitation of the labeled protein or complex moiety using antisera against the unlabeled binding partner (or labeled binding partner 25 with a distinguishable marker from that used on the second labeled protein or complex moiety), immunoaffinity chromatography, size exclusion chromatography, and gradient density centrifugation. In a preferred embodiment, the labeled binding partner is a small fragment or peptidomimetic that is not retained by a commercially available filter. 30 Upon binding, the labeled species is then unable to pass through the filter, providing for a simple assay of complex formation.

Methods commonly known in the art are used to label proteins. Suitable labeling methods include, but are not limited to, radiolabeling by incorporation of radiolabeled amino acids, e.g., ^3H -leucine or ^{35}S -methionine, radiolabeling by post-translational iodination with ^{125}I or ^{131}I using the chloramine T method, Bolton-Hunter reagents, etc., or labeling with ^{32}P using phosphorylase and inorganic radiolabeled phosphorous, biotin labeling with photobiotin-acetate and sunlamp exposure, etc.

10

5.8 THERAPEUTIC USES

The invention provides for treatment of disorders of cell fate or differentiation by administration of a therapeutic compound of the invention. Such therapeutic compounds (termed herein "Therapeutics") include: Delta cleavage peptides, Delta:Kuz and Dl^{EC} :Notch protein complexes and analogs and derivatives (including fragments) thereof (e.g., as described hereinabove); antibodies thereto (as described hereinabove); nucleic acids encoding the Delta cleavage peptides, analogs, or derivatives (e.g., as described hereinabove) as well as the protein complexes of the present invention; and Delta, Notch and Kuz antisense nucleic acids. In addition, such Therapeutics include soluble Delta peptides and derivatives and analogs thereof, antibodies thereto, nucleic acids encoding the soluble Delta peptides, derivatives, or analogs, and soluble Delta peptide antisense nucleic acids. In a particular embodiment, the Therapeutic is a peptide comprising a fragment of a Delta protein of about amino acid Cys_{516} to about amino acid Phe_{543} in human Delta (SEQ ID NO:10), of about amino acid Cys_{515} to about amino acid Phe_{543} in mouse Delta (SEQ ID NO:6), of about 20 amino acid Cys_{523} to about amino acid Phe_{551} in chick Delta (SEQ ID NO:7), of about amino acid Cys_{518} to about amino acid Phe_{544} in *Xenopus* Delta (SEQ ID NO:8), and the sequence of 25 30

about amino acid Cys₅₆₄ to about amino acid Ala₅₉₃ or Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9). In specific embodiments, the peptide comprises 25, 30, 35, 40, 50, 100, 150, 200 or 250 contiguous amino acids of a Delta protein. Antagonist

5 Therapeutics of the invention are those Therapeutics which antagonize, or inhibit, Delta function and/or Notch function (since Delta is a Notch ligand) and/or Kuz function (since Kuz binds to and proteolytically processes Delta). Such Antagonist Therapeutics are most preferably identified by use

10 of known convenient *in vitro* assays, e.g., based on their ability to inhibit binding of Delta to another protein (e.g., a Notch protein or a Kuz protein), or inhibit any known Notch or Delta or Kuz function as preferably assayed *in vitro* or in cell culture, although genetic assays (e.g., in *Drosophila*) may also be employed. In a preferred embodiment, the

15 Antagonist Therapeutic is a Delta cleavage peptide which mediates binding to Kuz, or an antibody thereto. In other specific embodiments, such an Antagonist Therapeutic is a nucleic acid capable of expressing a molecule comprising a Delta cleavage peptide which binds to Kuz, or a Delta

20 antisense nucleic acid (see Section 5.11 herein). It should be noted that preferably, suitable *in vitro* or *in vivo* assays, as described *infra*, should be utilized to determine the effect of a specific Therapeutic and whether its administration is indicated for treatment of the affected

25 tissue, since the developmental history of the tissue may determine whether an Antagonist or Agonist Therapeutic is desired.

In addition, the mode of administration, e.g., whether administered in soluble form or administered via its encoding nucleic acid for intracellular recombinant

30 expression, of the Delta cleavage peptide or derivative or protein complex or derivative can affect whether it acts as an agonist or antagonist.

The Agonist Therapeutics of the invention, as described *supra*, promote Delta function or Notch function or Kuz function. Such Agonist Therapeutics include but are not limited to proteins and derivatives comprising the portions 5 of Delta that mediate binding to Kuz, and nucleic acids encoding the foregoing (which can be administered to express their encoded products *in vivo*).

Further descriptions and sources of Therapeutics of the inventions are found in Sections 5.1 through 5.7 herein.

10 Molecules which retain, or alternatively inhibit, a desired Delta property, e.g., binding to Kuz, binding to an intracellular ligand, can be used therapeutically as inducers, or inhibitors, respectively, of such property and its physiological correlates. In a specific embodiment, a peptide (e.g., in the range of 6-50 or 100-200 amino acids; 15 and particularly of about 25, 30, 35, 50, 100 or 150 amino acids) containing the sequence of a portion of Delta which binds to Kuz is used to antagonize Delta or Notch function. In a specific embodiment, such an Antagonist Therapeutic is used to treat or prevent human or other malignancies 20 associated with increased Notch expression (e.g., cervical cancer, colon cancer, breast cancer, squamous adenocarcinomas (see *infra*)). Derivatives or analogs of Delta can be tested for the desired activity by procedures known in the art, including but not limited to the assays described in the 25 examples *infra*. In one specific embodiment, peptide libraries can be screened to select a peptide with the desired activity; such screening can be carried out by assaying, e.g., for binding to Kuz.

Other Therapeutics include molecules that bind to a Kuz. Thus, the invention also provides a method for 30 identifying such molecules. Such molecules can be identified by a method comprising contacting a plurality of molecules (e.g., in a peptide library, or combinatorial chemical

library) with the Kuz protein under conditions conducive to binding, and recovering any molecules that bind to the Kuz protein.

The Agonist and Antagonist Therapeutics of the 5 invention have therapeutic utility for disorders of cell fate. The Agonist Therapeutics are administered therapeutically (including prophylactically): (1) in diseases or disorders involving an absence or decreased (relative to normal, or desired) levels of Notch or Delta or Kuz function, for example, in patients where Delta protein is lacking, 10 genetically defective, biologically inactive or underactive, or underexpressed; and (2) in diseases or disorders wherein *in vitro* (or *in vivo*) assays (see *infra*) indicate the utility of Delta agonist administration. The absence or decreased levels in Notch or Delta or Kuz function can be readily 15 detected, e.g., by obtaining a patient tissue sample (e.g., from biopsy tissue) and assaying it *in vitro* for protein levels, structure and/or activity of the expressed Notch or Delta or Kuz protein. Many methods standard in the art can be thus employed, including but not limited to immunoassays 20 to detect and/or visualize Notch or Delta or Kuz protein (e.g., Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect Notch or Delta or Kuz expression by detecting and/or 25 visualizing respectively Notch or Delta or Kuz mRNA (e.g., Northern assays, dot blots, *in situ* hybridization, etc.)

In vitro assays which can be used to determine whether administration of a specific Agonist Therapeutic or Antagonist Therapeutic is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in 30 culture, and exposed to or otherwise administered a Therapeutic, and the effect of such Therapeutic upon the tissue sample is observed. In one embodiment, where the

patient has a malignancy, a sample of cells from such malignancy is plated out or grown in culture, and the cells are then exposed to a Therapeutic. A Therapeutic which inhibits survival or growth of the malignant cells (e.g., by 5 promoting terminal differentiation) is selected for therapeutic use *in vivo*. Many assays standard in the art can be used to assess such survival and/or growth; for example, cell proliferation can be assayed by measuring ^3H -thymidine incorporation, by direct cell count, by detecting changes in transcriptional activity of known genes such as proto- 10 oncogenes (e.g., *fos*, *myc*) or cell cycle markers; cell viability can be assessed by trypan blue staining, differentiation can be assessed visually based on changes in morphology, etc. In a specific aspect, the malignant cell cultures are separately exposed to (1) an Agonist 15 Therapeutic, and (2) an Antagonist Therapeutic; the result of the assay can indicate which type of Therapeutic has therapeutic efficacy.

In another embodiment, a Therapeutic is indicated for use which exhibits the desired effect, inhibition or 20 promotion of cell growth, upon a patient cell sample from tissue having or suspected of having a hyper- or hypoproliferative disorder, respectively. Such hyper- or hypoproliferative disorders include but are not limited to those described in Sections 5.8.1 through 5.8.3 *infra*.

25 In another specific embodiment, a Therapeutic is indicated for use in treating nerve injury or a nervous system degenerative disorder (see Section 5.8.2) which exhibits *in vitro* promotion of nerve regeneration/neurite extension from nerve cells of the affected patient type.

In addition, administration of an Antagonist 30 Therapeutic of the invention is also indicated in diseases or disorders determined or known to involve a Notch or Delta or Kuz dominant activated phenotype ("gain of function"

mutations.) Administration of an Agonist Therapeutic is indicated in diseases or disorders determined or known to involve a Notch or Delta or Kuz dominant negative phenotype ("loss of function" mutations). The functions of various 5 structural domains of the Notch protein have been investigated *in vivo*, by ectopically expressing a series of *Drosophila* Notch deletion mutants under the hsp70 heat-shock promoter, as well as eye-specific promoters (see Rebay et al., 1993, *Cell* 74:319-329). Two classes of dominant 10 phenotypes were observed, one suggestive of Notch loss-of function mutations and the other of Notch gain-of-function mutations. Dominant "activated" phenotypes resulted from overexpression of a protein lacking most extracellular sequences, while dominant "negative" phenotypes resulted from overexpression of a protein lacking most intracellular 15 sequences. The results indicated that Notch functions as a receptor whose extracellular domain mediates ligand-binding, resulting in the transmission of developmental signals by the cytoplasmic domain.

In various specific embodiments, *in vitro* assays 20 can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a Therapeutic has a desired effect upon such cell types.

In another embodiment, cells of a patient tissue sample suspected of being pre-neoplastic are similarly plated 25 out or grown *in vitro*, and exposed to a Therapeutic. The Therapeutic which results in a cell phenotype that is more normal (i.e., less representative of a pre-neoplastic state, neoplastic state, malignant state, or transformed phenotype) is selected for therapeutic use. Many assays standard in the art can be used to assess whether a pre-neoplastic state, 30 neoplastic state, or a transformed or malignant phenotype, is present. For example, characteristics associated with a transformed phenotype (a set of *in vitro* characteristics

associated with a tumorigenic ability *in vivo*) include a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar 5 transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton surface protein, etc. (see Luria et al., 1978, *General Virology*, 3d Ed., John Wiley & Sons, New York pp. 436-446).

In other specific embodiments, the *in vitro* assays 10 described *supra* can be carried out using a cell line, rather than a cell sample derived from the specific patient to be treated, in which the cell line is derived from or displays characteristic(s) associated with the malignant, neoplastic or pre-neoplastic disorder desired to be treated or prevented, or is derived from the neural or other cell type 15 upon which an effect is desired, according to the present invention.

The Antagonist Therapeutics are administered therapeutically (including prophylactically): (1) in diseases or disorders involving increased (relative to normal, or 20 desired) levels of Notch or Delta or Kuz function, for example, where the Notch or Delta or Kuz protein is overexpressed or overactive; and (2) in diseases or disorders wherein *in vitro* (or *in vivo*) assays indicate the utility of Delta antagonist administration. The increased levels of 25 Notch or Delta or Kuz function can be readily detected by methods such as those described above, by quantifying protein and/or RNA. *In vitro* assays with cells of patient tissue sample or the appropriate cell line or cell type, to determine therapeutic utility, can be carried out as described above.

5.8.1 MALIGNANCIES

Malignant and pre-neoplastic conditions which can be tested as described *supra* for efficacy of intervention with Antagonist or Agonist Therapeutics, and which can be 5 treated upon thus observing an indication of therapeutic utility, include but are not limited to those described below in Sections 5.8.1 and 5.9.1.

Malignancies and related disorders, cells of which type can be tested *in vitro* (and/or *in vivo*), and upon 10 observing the appropriate assay result, treated according to the present invention, include but are not limited to those listed in Table 1 (for a review of such disorders, see Fishman et al., 1985, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia):

15

TABLE 1
MALIGNANCIES AND RELATED DISORDERS

	Leukemia
	acute leukemia
20	acute lymphocytic leukemia
	acute myelocytic leukemia
	myeloblastic
	promyelocytic
	myelomonocytic
	monocytic
	erythroleukemia
	chronic leukemia
25	chronic myelocytic (granulocytic) leukemia
	chronic lymphocytic leukemia
	Polycythemia vera
	Lymphoma
	Hodgkin's disease
	non-Hodgkin's disease
	Multiple myeloma
	Waldenström's macroglobulinemia
30	Heavy chain disease
	Solid tumors
	sarcomas and carcinomas
	fibrosarcoma
	myxosarcoma

5	liposarcoma chondrosarcoma osteogenic sarcoma chordoma angiosarcoma endotheliosarcoma lymphangiosarcoma lymphangioendotheliosarcoma synovioma mesothelioma Ewing's tumor leiomyosarcoma rhabdomyosarcoma colon carcinoma pancreatic cancer breast cancer ovarian cancer prostate cancer squamous cell carcinoma basal cell carcinoma adenocarcinoma sweat gland carcinoma sebaceous gland carcinoma papillary carcinoma papillary adenocarcinomas cystadenocarcinoma medullary carcinoma bronchogenic carcinoma renal cell carcinoma hepatoma
10	
15	
20	bile duct carcinoma choriocarcinoma seminoma embryonal carcinoma Wilms' tumor cervical cancer testicular tumor lung carcinoma
25	small cell lung carcinoma bladder carcinoma epithelial carcinoma glioma astrocytoma medulloblastoma craniopharyngioma ependymoma
30	pinealoma hemangioblastoma acoustic neuroma oligodendroglioma menangioma

melanoma
neuroblastoma
retinoblastoma

5 In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and dysplasias) are treated or prevented in epithelial tissues such as those in the cervix, esophagus, and lung.

10 Malignancies of the colon and cervix exhibit increased expression of human Notch relative to such non-malignant tissue (see PCT Publication no. WO 94/07474 published April 14, 1994, incorporated by reference herein in its entirety). Thus, in specific embodiments, malignancies or premalignant changes of the colon or cervix are treated or prevented by administering an effective amount of an
15 Antagonist Therapeutic, e.g., a Delta cleavage peptide, that antagonizes Notch function. The presence of increased Notch expression in colon, and cervical cancer suggests that many more cancerous and hyperproliferative conditions exhibit upregulated Notch. Thus, in specific embodiments, various
20 cancers, e.g., breast cancer, squamous adenocarcinoma, seminoma, melanoma, and lung cancer, and premalignant changes therein, as well as other hyperproliferative disorders, can be treated or prevented by administration of an Antagonist Therapeutic that antagonizes Notch function.

25

5.8.2 NERVOUS SYSTEM DISORDERS

Nervous system disorders, involving cell types which can be tested as described *supra* for efficacy of intervention with Antagonist or Agonist Therapeutics, and
30 which can be treated upon thus observing an indication of therapeutic utility, include but are not limited to nervous system injuries, and diseases or disorders which result in either a disconnection of axons, a diminution or degeneration

of neurons, or demyelination. Nervous system lesions which may be treated in a patient (including human and non-human mammalian patients) according to the invention include but are not limited to the following lesions of either the 5 central (including spinal cord, brain) or peripheral nervous systems:

- (i) traumatic lesions, including lesions caused by physical injury or associated with surgery, for example, lesions which sever a portion of the nervous system, or compression injuries;
- 10 (ii) ischemic lesions, in which a lack of oxygen in a portion of the nervous system results in neuronal injury or death, including cerebral infarction or ischemia, or spinal cord infarction or ischemia;
- 15 (iii) malignant lesions, in which a portion of the nervous system is destroyed or injured by malignant tissue which is either a nervous system associated malignancy or a malignancy derived from non-nervous system tissue;
- 20 (iv) infectious lesions, in which a portion of the nervous system is destroyed or injured as a result of infection, for example, by an abscess or associated with infection by human immunodeficiency virus, herpes zoster, or herpes simplex virus or with Lyme disease, tuberculosis, syphilis;
- 25 (v) degenerative lesions, in which a portion of the nervous system is destroyed or injured as a result of a degenerative process including but not limited to degeneration associated with Parkinson's disease, Alzheimer's disease, Huntington's chorea, or amyotrophic lateral sclerosis;

5 (vi) lesions associated with nutritional diseases or disorders, in which a portion of the nervous system is destroyed or injured by a nutritional disorder or disorder of metabolism including but not limited to, vitamin B12 deficiency, folic acid deficiency, Wernicke disease, tobacco-alcohol amblyopia, Marchiafava-Bignami disease (primary degeneration of the corpus callosum), and alcoholic cerebellar degeneration;

10 (vii) neurological lesions associated with systemic diseases including but not limited to diabetes (diabetic neuropathy, Bell's palsy), systemic lupus erythematosus, carcinoma, or sarcoidosis;

15 (viii) lesions caused by toxic substances including alcohol, lead, or particular neurotoxins; and (ix) demyelinated lesions in which a portion of the nervous system is destroyed or injured by a demyelinating disease including but not limited to multiple sclerosis, human immunodeficiency virus-associated myelopathy, transverse myelopathy or various etiologies, progressive multifocal leukoencephalopathy, and central pontine myelinolysis.

20 Therapeutics which are useful according to the invention for treatment of a nervous system disorder may be selected by testing for biological activity in promoting the survival or differentiation of neurons (see also Section 5.8). For example, and not by way of limitation, Therapeutics which elicit any of the following effects may be

25 useful according to the invention:

(i) increased survival time of neurons in culture;

- (ii) increased sprouting of neurons in culture or *in vivo*;
- (iii) increased production of a neuron-associated molecule in culture or *in vivo*, e.g., choline acetyltransferase or acetylcholinesterase with respect to motor neurons; or
- 5 (iv) decreased symptoms of neuron dysfunction *in vivo*.

Such effects may be measured by any method known in the art.

10 In preferred, non-limiting embodiments, increased survival of neurons may be measured by the method set forth in Arakawa et al. (1990, *J. Neurosci.* 10:3507-3515); increased sprouting of neurons may be detected by methods set forth in Pestronk et al. (1980, *Exp. Neurol.* 70:65-82) or Brown et al. (1981, *Ann. Rev. Neurosci.* 4:17-42); increased production of neuron-15 associated molecules may be measured by bioassay, enzymatic assay, antibody binding, Northern blot assay, etc., depending on the molecule to be measured; and motor neuron dysfunction may be measured by assessing the physical manifestation of motor neuron disorder, e.g., weakness, motor neuron 20 conduction velocity, or functional disability.

In a specific embodiments, motor neuron disorders that may be treated according to the invention include but are not limited to disorders such as infarction, infection, exposure to toxin, trauma, surgical damage, degenerative 25 disease or malignancy that may affect motor neurons as well as other components of the nervous system, as well as disorders that selectively affect neurons such as amyotrophic lateral sclerosis, and including but not limited to progressive spinal muscular atrophy, progressive bulbar palsy, primary lateral sclerosis, infantile and juvenile 30 muscular atrophy, progressive bulbar paralysis of childhood (Fazio-Londe syndrome), poliomyelitis and the post polio

syndrome, and Hereditary Motorsensory Neuropathy (Charcot-Marie-Tooth Disease).

5.8.3 TISSUE REPAIR AND REGENERATION

5 In another embodiment of the invention, a Therapeutic of the invention is used for promotion of tissue regeneration and repair, including but not limited to treatment of benign dysproliferative disorders. Specific embodiments are directed to treatment of cirrhosis of the 10 liver (a condition in which scarring has overtaken normal liver regeneration processes), treatment of keloid (hypertrophic scar) formation (disfiguring of the skin in which the scarring process interferes with normal renewal), psoriasis (a common skin condition characterized by excessive proliferation of the skin and delay in proper cell fate 15 determination), and baldness (a condition in which terminally differentiated hair follicles (a tissue rich in Notch) fail to function properly). In another embodiment, a Therapeutic of the invention is used to treat degenerative or traumatic disorders of the sensory epithelium of the inner ear.

20

5.9 PROPHYLACTIC USES

5.9.1 MALIGNANCIES

The Therapeutics of the invention can be administered to prevent progression to a neoplastic or 25 malignant state, including but not limited to those disorders listed in Table 1. Such administration is indicated where the Therapeutic is shown in assays, as described *supra*, to have utility for treatment or prevention of such disorder. Such prophylactic use is indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in 30 particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see

Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-79.) Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. As but one example, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladder.

Alternatively or in addition to the presence of abnormal cell growth characterized as hyperplasia, metaplasia, or dysplasia, the presence of one or more characteristics of a transformed phenotype, or of a malignant phenotype, displayed *in vivo* or displayed *in vitro* by a cell sample from a patient, can indicate the desirability of prophylactic/therapeutic administration of a Therapeutic of the invention. As mentioned *supra*, such characteristics of a transformed phenotype include morphology changes, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton cell surface

protein, etc. (see also *id.*, at pp. 84-90 for characteristics associated with a transformed or malignant phenotype).

In a specific embodiment, leukoplakia, a benign-appearing hyperplastic or dysplastic lesion of the 5 epithelium, or Bowen's disease, a carcinoma *in situ*, are pre-neoplastic lesions indicative of the desirability of prophylactic intervention.

In another embodiment, fibrocystic disease (cystic hyperplasia, mammary dysplasia, particularly adenosis (benign 10 epithelial hyperplasia)) is indicative of the desirability of prophylactic intervention.

In other embodiments, a patient which exhibits one or more of the following predisposing factors for malignancy is treated by administration of an effective amount of a Therapeutic: a chromosomal translocation associated with a 15 malignancy (e.g., the Philadelphia chromosome for chronic myelogenous leukemia, t(14;18) for follicular lymphoma, etc.), familial polyposis or Gardner's syndrome (possible forerunners of colon cancer), benign monoclonal gammopathy (a possible forerunner of multiple myeloma), and a first degree 20 kinship with persons having a cancer or precancerous disease showing a Mendelian (genetic) inheritance pattern (e.g., familial polyposis of the colon, Gardner's syndrome, hereditary exostosis, polyendocrine adenomatosis, medullary thyroid carcinoma with amyloid production and 25 pheochromocytoma, Peutz-Jeghers syndrome, neurofibromatosis of Von Recklinghausen, retinoblastoma, carotid body tumor, cutaneous melanocarcinoma, intraocular melanocarcinoma, xeroderma pigmentosum, ataxia telangiectasia, Chediak-Higashi syndrome, albinism, Fanconi's aplastic anemia, and Bloom's syndrome; see Robbins and Angell, 1976, *Basic Pathology*, 2d 30 Ed., W.B. Saunders Co., Philadelphia, pp. 112-113) etc.)

In another specific embodiment, an Antagonist Therapeutic of the invention is administered to a human

patient to prevent progression to breast, colon, or cervical cancer.

5.9.2 OTHER DISORDERS

5 In other embodiments, a Therapeutic of the invention can be administered to prevent a nervous system disorder described in Section 5.8.2, or other disorder (e.g., liver cirrhosis, psoriasis, keloids, baldness) described in Section 5.8.3.

10

5.10 DEMONSTRATION OF THERAPEUTIC OR PROPHYLACTIC UTILITY

The Therapeutics of the invention can be tested *in vivo* for the desired therapeutic or prophylactic activity. For example, such compounds can be tested in suitable animal 15 model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used.

20

5.11 USE OF ANTISENSE OLIGONUCLEOTIDES FOR SUPPRESSION OF DELTA ACTIVATION OR DELTA:KUZ OR D^{LE^c}:NOTCH COMPLEX ACTIVITY OR FORMATION

In a specific embodiment of the present invention, Delta cleavage peptide, Delta, Kuz, Notch, and Delta:Kuz or D^{LE^c}:Notch complex activity and/or formation, is inhibited by 25 use of antisense nucleic acids for Delta, Notch and/or Kuz. The present invention provides the therapeutic or prophylactic use of nucleic acids of at least six nucleotides that are antisense to a gene or cDNA encoding Delta, Notch and/or Kuz, or a portion thereof. An "antisense" nucleic acid as used herein refers to a nucleic acid capable of 30 hybridizing to a portion of a Delta, Notch or Kuz RNA (preferably mRNA) by virtue of some sequence complementarily. The antisense nucleic acid may be complementary to a coding

and/or noncoding region of a Delta, Notch or Kuz mRNA. Such antisense nucleic acids that inhibit Delta cleavage peptide activity or Delta:Kuz complex formation or activity or Dl^{sc}:Notch complex formation or activity have utility as 5 Therapeutics, and can be used in the treatment or prevention of disorders as described, *supra*.

The antisense nucleic acids of the invention can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA, or a modification or derivative thereof, which 10 can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

In another embodiment, the present invention is directed to a method for inhibiting the expression of Delta cleavage peptide nucleic acid sequences, in a prokaryotic or 15 eukaryotic cell, comprising providing the cell with an effective amount of a composition comprising an antisense nucleic acid of Delta cleavage peptide, or a derivative thereof, of the invention.

The antisense nucleic acids are of at least six 20 nucleotides and are preferably oligonucleotides, ranging from 6 to about 200 nucleotides. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or 25 chimeric mixtures, or derivatives or modified versions thereof, and either single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents facilitating transport across the cell membrane (see, e.g., 30 Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; International Patent Publication No. WO 88/09810)

or blood-brain barrier (see, e.g., International Patent Publication No. WO 89/10134), hybridization-triggered cleavage agents (see, e.g., Krol et al., 1988, BioTechniques 6:958-976), or intercalating agents (see, e.g., Zon, 1988, 5 Pharm. Res. 5:539-549).

In a preferred aspect of the invention, a Delta cleavage peptide antisense oligonucleotide is provided, preferably as single-stranded DNA. The oligonucleotide may be modified at any position in its structure with constituents generally known in the art.

10 The antisense oligonucleotides may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil, 15 5-carboxymethylaminomethyl-2-thio-uridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 20 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5N-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methyl-thio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, 25 queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

30 In another embodiment, the oligonucleotide comprises at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal, or an analog of the foregoing.

In yet another embodiment, the oligonucleotide is a 2- α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA 10 in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641).

The oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization-triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer (such as are commercially 20 available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligo-nucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-25 7451), etc.

In a specific embodiment, the antisense oligonucleotides comprise catalytic RNAs, or ribozymes (see, e.g., International Patent Publication No. WO 90/11364; Sarver et al., 1990, Science 247:1222-1225). In another 30 embodiment, the oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res.

15:6131-6148), or a chimeric RNA-DNA analog (Inoue et al., 1987, FEBS Lett. 215:327-330).

In an alternative embodiment, the antisense nucleic acids of the invention are produced intracellularly by 5 transcription from an exogenous sequence. For example, a vector can be introduced in vivo such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector can remain episomal or become 10 chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art to be capable of replication and expression in mammalian cells. Expression of the sequences encoding the 15 antisense RNAs can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in 20 the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), etc. 25

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a Delta, Notch or Kuz gene, preferably a human Delta, Notch or Kuz gene. However, absolute complementarily, although preferred, is not required. A 30 sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarily to be able to hybridize with the RNA, forming

a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarily and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

The antisense nucleic acid can be used to treat (or prevent) disorders of a cell type that expresses, or preferably overexpresses, the Delta cleavage peptide or the Delta:Kuz complex or the D^{lec}:Notch complex. In a preferred embodiment, a single-stranded Delta, Notch or Kuz DNA antisense oligonucleotide, both single-stranded Delta, Notch and Kuz antisense oligonucleotides, or a single-stranded Delta:Kuz DNA antisense fusion sequence, is used.

Cell types that express or overexpress Delta, Notch and/or Kuz RNA can be identified by various methods known in the art. Such methods include, but are not limited to, hybridization with Delta-, Notch- and Kuz-specific nucleic acids (e.g., by Northern blot hybridization, dot blot hybridization, or *in situ* hybridization), or by observing the ability of RNA from the cell type to be translated *in vitro* into Delta or Kuz by immunohistochemistry, Western blot analysis, ELISA, etc. In a preferred aspect, primary tissue from a patient can be assayed for Delta, Notch and/or Kuz expression prior to treatment, e.g., by immunocytochemistry, *in situ* hybridization, or any number of methods to detect protein or mRNA expression.

Pharmaceutical compositions of the invention (see Section 5.7, *infra*), comprising an effective amount of an

antisense nucleic acid in a pharmaceutically acceptable carrier can be administered to a patient having a disease or disorder that is of a type that expresses or overexpresses, for example a Delta:Kuz complex.

5 The amount of an antisense nucleic acid that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where possible, it is desirable to determine the 10 antisense cytotoxicity in vitro, and then in useful animal model systems, prior to testing and use in humans.

In a specific embodiment, pharmaceutical compositions comprising Delta and Kuz antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it 15 may be useful to use such compositions to achieve sustained release of the Delta and/or Kuz antisense nucleic acids. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable central nervous system cell types (Leonetti et al., 1990, 20 Proc. Natl. Acad. Sci. U.S.A. 87:2448-2451; Renneisen et al., 1990, J. Biol. Chem. 265:16337-16342).

5.12 THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment (and 25 prophylaxis) by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to animals such as cows, pigs, chickens, etc., and is preferably 30 a mammal, and most preferably human.

Various delivery systems are known and can be used to administer a Therapeutic of the invention, e.g.,

encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a Therapeutic nucleic acid as 5 part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or 10 mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention 15 into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an 20 inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention 25 locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, 30 non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or

former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment, the Therapeutic can be delivered in a vesicle, in particular a liposome (see Langer, 5 Science 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

In yet another embodiment, the Therapeutic can be delivered in a controlled release system. In one embodiment, 10 a pump may be used (see Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see 15 Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also Levy et al., Science 228:190 (1985); During et al., 20 Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical 25 Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

In a specific embodiment where the Therapeutic is a nucleic acid encoding a protein Therapeutic, the nucleic acid 30 can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that

it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biostatic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid Therapeutic can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

In specific embodiments directed to treatment or prevention of particular disorders, preferably the following forms of administration are used:

<u>15 Disorder</u>	<u>Preferred Forms of Administration</u>
Cervical cancer	Topical
Gastrointestinal cancer	Oral; intravenous
Lung cancer	Inhaled; intravenous
Leukemia	Intravenous; extracorporeal
20 Metastatic carcinomas	Intravenous; oral
Brain cancer	Targeted; intravenous; intrathecal
Liver cirrhosis	Oral; intravenous
Psoriasis	Topical
Keloids	Topical
Baldness	Topical
25 Spinal cord injury	Targeted; intravenous; intrathecal
Parkinson's disease	Targeted; intravenous; intrathecal
Motor neuron disease	Targeted; intravenous; intrathecal
Alzheimer's disease	Targeted; intravenous; intrathecal

30 The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically

acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia 5 for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral 10 oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable 15 pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, 20 can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, 25 with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. 30 Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to

provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection.

Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard

clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the 5 seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are 10 generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient 15 in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of 20 the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

25

5.13 DIAGNOSTIC UTILITY

Delta cleavage peptides, soluble Delta peptides, 30 analogs, derivatives, and subsequences thereof, Delta cleavage peptide encoding nucleic acids (and sequences complementary thereto), soluble Delta peptide encoding nucleic acids (and sequences complementary thereto), anti-Delta cleavage peptide antibodies, anti-soluble Delta peptide

antibodies, and anti-Delta:Kuz and anti-Dl^{EC}:Notch complex antibodies have uses in diagnostics. Such molecules can be used in assays, such as immunoassays, to detect, prognose, diagnose, or monitor various conditions, diseases, and

5 disorders affecting Delta cleavage peptide expression, or monitor the treatment thereof. In a particular example, such an immunoassay is carried out by a method comprising contacting a sample derived from a patient with an anti-Delta cleavage peptide antibody under conditions such that

10 immunospecific binding can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. In a specific aspect, such binding of antibody, in tissue sections, preferably in conjunction with binding of anti-Kuz or anti-Notch antibody can be used to detect aberrant Delta, Notch and/or Kuz localization or aberrant levels of Dl^{EC}:Notch

15 or Delta-Kuz colocalization in a disease state. In a specific embodiment, antibody to Delta cleavage peptide can be used to assay in a patient tissue or serum sample for the presence of Delta cleavage peptide where an aberrant level of Delta cleavage peptide is an indication of a diseased

20 condition. Aberrant levels of Delta binding ability in an endogenous Notch or Kuz protein, or aberrant levels of binding ability to Kuz (or other Delta ligand, e.g., Notch) in an endogenous Delta cleavage peptide may be indicative of a disorder of cell fate (e.g., cancer, etc.) By "aberrant

25 levels," is meant increased or decreased levels relative to that present, or a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having the disorder.

The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems

30 using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin

reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few.

5 *Delta, Notch and Kuz genes and related nucleic acid sequences and subsequences, including complementary sequences, and other toporythmic gene sequences, can also be used in hybridization assays.* *Delta, Notch and Kuz nucleic acid sequences, or subsequences thereof comprising about at least 8 nucleotides, can be used as hybridization probes.*

10 *Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with aberrant changes in Delta expression and/or activity as described supra.* In particular, such a hybridization assay is carried out by a method comprising

15 *contacting a sample containing nucleic acid with a nucleic acid probe capable of hybridizing to Delta, Notch or Kuz DNA or RNA, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization.*

 Additionally, since Delta binds to Notch and Kuz, 20 Delta or a binding portion thereof can be used to assay for the presence and/or amounts of Notch or Kuz in a sample, e.g., in screening for malignancies which exhibit increased Notch expression such as colon and cervical cancers.

25 5.14 ANIMAL MODELS

 The present invention also provides animal models. In one embodiment, animal models for diseases and disorders involving Delta cleavage peptide, soluble Delta peptide, and Delta:Kuz and Dl^{EC}:Notch complexes are provided. These include, but are not limited to, disorders of cell fate and 30 differentiation such as cancer. Such animals can be initially produced by promoting homologous recombination or insertional mutagenesis between Delta, Notch and Kuz genes in

the chromosome, and exogenous *Delta*, *Notch* and *Kuz* genes that have been rendered biologically inactive or deleted (preferably by insertion of a heterologous sequence, e.g., an antibiotic resistance gene). In a preferred aspect, 5 homologous recombination is carried out by transforming embryo-derived stem (ES) cells with a vector containing, e.g., the insertionally inactivated *Delta* and *Kuz* gene, such that homologous recombination occurs, followed by injecting the transformed ES cells into a blastocyst, and implanting 10 the blastocyst into a foster mother, followed by the birth of the chimeric animal ("knockout animal") in which a *Delta* and/or *Kuz* gene has been inactivated or deleted (Capecchi, 1989, Science 244:1288-1292). The chimeric animal can be bred to produce additional knockout animals. Such animals can be mice, hamsters, sheep, pigs, cattle, etc., and are 15 preferably non-human mammals. In a specific embodiment, a knockout mouse is produced.

Such knockout animals are expected to develop, or be predisposed to developing, diseases or disorders involving, but not restricted to, disorders of cell fate and 20 differentiation, etc., and thus, can have use as animal models of such diseases and disorders, e.g., to screen for or test molecules (e.g., potential Therapeutics) for disorders of cell fate and differentiation, and other diseases.

In a different embodiment of the invention, 25 transgenic animals that have incorporated and express (or overexpress or mis-express) a functional *Delta* and/or *Kuz* gene, e.g. by introducing the *Delta* and *Kuz* genes under the control of a heterologous promoter (i.e., a promoter that is not the native *Delta* or *Kuz* promoter) that either overexpresses the protein or proteins, or expresses them in 30 tissues not normally expressing the complexes or proteins, can have use as animal models of diseases and disorders characterized by elevated levels of *Delta*:*Kuz* complexes.

Such animals can be used to screen or test molecules for the ability to treat or prevent the diseases and disorders cited *supra*.

In one embodiment, the present invention provides a recombinant non-human animal in which both an endogenous Delta gene and an endogenous Kuz have been deleted or inactivated by homologous recombination or insertional mutagenesis of said animal or an ancestor thereof. In another embodiment, the invention provides a recombinant non-human animal containing both a Delta gene and a Kuz gene in which the Delta gene is under the control of a promoter that is not the native Kuz gene promoter and the Kuz gene is under the control of a promoter that is not the native Kuz gene promoter. In a specific embodiment, the invention provides a recombinant non-human animal containing a transgene comprising a nucleic acid sequence encoding a chimeric protein comprising a Delta cleavage peptide of at least 6 amino acids fused via a covalent bond to a fragment of Kuz protein of at least 6 amino acids.

20 6. THE NOTCH LIGAND DELTA IS CLEAVED BY THE
DISINTEGRIN METALLOPROTEASE KUZBANIAN

The Notch signaling pathway defines an evolutionary conserved cell interaction mechanism which throughout development controls the fate of cells by modulating their response to developmental signals (Artavanis-Tsakonas et al., 25 1995, Science 268:225-232; Fleming et al., 1998, Trends in Cell Biology 7:437-441). The Notch receptor is cleaved in the trans-Golgi network as it traffics towards the plasma membrane eventually forming a ligand competent, heterodimeric molecule (Blaumueller et al., 1997, Cell 90:281-291). Both 30 known ligands, Delta and Serrate are thought to act as transmembrane proteins interacting via their extracellular domains with the receptor expressed on adjacent cells

(Fleming et al., 1998, Trends in Cell Biology 7:437-441; Muskavitch, 1994, Developmental Biology 166:415-430). Given the similar phenotypes between loss of Notch signaling and loss of function mutations in the Kuzbanian (Kuz) gene, a 5 gene encoding a putative member of the ADAM family of metalloproteases (Rooke et al., 1996, Nature 273:1227-1231), it has been suggested that Kuz may be involved in the cleavage of the Notch receptor (Pan and Rubin, 1997, Cell 90:271-280. This hypothesis is not corroborated by recent 10 biochemical studies which indicate that the functionally crucial cleavage of Notch in the trans Golgi network is catalyzed by a furin-like convertase (Logeat, et al., 1998, Proc. Nat. Acad. Sci. USA 95:8108-8112). Consistent with this, furin is known to act in this sub-cellular compartment, as opposed to ADAM proteases, such as Kuz, which are thought 15 to act on the cell surface (Wolfsberg et al., 1995, Journal of Cell Biology 131:275-278).

A genetic screen aimed in identifying modifiers of the phenotypes associated with the constitutive expression of a dominant negative transgene of Kuz (KuzDN) in developing 20 imaginal discs, has uncovered Delta as an interacting gene (Wu et al., unpublished observation). Flies expressing this dominant negative construct, even though they also carry a wild type complement of Kuz become semi-lethal when heterozygous for a loss of function Delta mutation (Xu et 25 al., unpublished observation). In contrast, Delta duplications rescue the phenotypes associated with KuzDN (Figures 6A-6F). KuzDN flies display extra vein material, especially deltas, at the ends of the longitudinal veins, wing notching (observed with a low penetrance), extra bristles on the notum, and have small, rough eyes (Figures 6A 30 and 6E). When KuzDN flies carry three, as opposed to the normal two, copies of wild type Notch, the bristle and eye phenotype are not affected (Xu et al., unpublished

observation), nor are the vein deltas altered (Figure 6D). On the other hand, the KuzDN phenotypes are effectively suppressed by Delta duplications (Figures 6B and 6F). Indicating that a higher copy number of Delta molecules is 5 capable of overriding the effect of the KuzDN construct.

The interaction between Delta and Kuz was further explored by examining the relationship between the protein products of their respective genes. A monoclonal antibody was raised against an extracellular Delta epitope generated by using a fusion protein generated by using a PCR product of 10 the entire extracellular domain of Drosophila Delta using the primers 5' GAGTTGCGCCTGAAGTACTT 3' (SEQ ID NO:14) and 5' GGTGCGCTCCATATTGGTGGG 3' (SEQ ID NO:15) and subsequent cloning into the SmaI site of pGEX3 and StuI site of pMAL. A monoclonal cell line (C594.9B, designated "9B") was created 15 by standard protocols and screening of hybridoma supernatants was done by immunostaining of Delta expressing S2 cells. Ascites fluid was made and used at 1/3000-1/10,000 dilution for western blotting followed by detection with peroxidase labelled anti-mouse antibody and chemiluminescent development 20 with a luminol substrate (see Rand et al., 1997, Protein Science 6:2059-2071). Using this antibody, the Delta antigen in S2 cells, which stably express full length Delta, was examined (Figure 7A). S2 cells are known to express wild type Kuz endogenously (Pan and Rubin, 1997, Cell 90:271-280). The presence of an immunoreactive fragment in the culture 25 media that migrated faster than full length Delta was observed exclusively in the media. It is noted that this fragment, as with full length Delta, was fully 40-fold more immunoreactive with 9B under non-reducing conditions. Full length Delta is associated with the cell pellet whereas, this 30 fragment is almost exclusively in the media suggesting it is a soluble, proteolytic fragment derived from full length Delta (herein referred to as "D^{EC}"). The size of this

fragment under reducing conditions is approximately 67,000 Daltons, consistent the extracellular domain of Delta, which is estimated to be 65,000 Daltons (Figure 7D). D₁^{EC} was subsequently affinity purified from the culture medium and 5 subjected to amino acid sequence analysis to determine the N-terminal amino acid sequence. Briefly, *Drosophila* S2 cells expressing Delta were induced with 0.7 mM CuSO₄ in serum free media for two days and the media was collected and precipitated with 70% ammonium sulfate. The precipitate was 10 collected by centrifugation and subsequently resuspended and dialyzed against 20 mM HEPES, 150 mM NaCl, 2 mM CaCl₂, pH 7.4. This sample was passed over Sepharose beads coupled with monoclonal antibody 9B, washed with 1.0 M NaCl and eluted with 25 mM glycine, pH 2.8 and immediately neutralized with 1.0 M Tris-HCl. N-terminal amino acid analysis was 15 performed with an Applied Biosystems gas phase amino acid sequencer.

The amino acid sequence of D₁^{EC} is consistent with the predicted polypeptide processing site and is conserved among the *Drosophila*, *Xenopus* and human Delta homologs 20 (Figure 7E).

It is concluded that full length Delta in S2 cells is cleaved at the surface to release a fragment containing most or all of the extracellular domain of Delta (D₁^{EC}). Western blot analysis of *Drosophila* embryos reveals the 25 existence of both full length Delta (D₁^{FL}) and a fragment with the same mobility as D₁^{EC} implicating this same Delta derived product is present *in vivo* (Figure 7B). It is noted that between D₁^{FL} and D₁^{EC} additional potentially transient proteolytic products are detectable with the 9B antibody (Figure 7B, lane "10 embryos" and Figure 8D, lane "kuz +/-").

30 The possibility that the generation of D₁^{EC} can be influenced by Kuz was examined by cotransfection experiments in S2 cells which, as mentioned earlier, are known to express

wild type Kuz endogenously (Pan and Rubin, 1997, Cell 90:271-280). In transient transfections, cotransfection of Delta with Kuz showed a remarkable increase in the Dl^{EC} fragment in the culture medium compared to Delta transfection alone 5 (Figure 8A). This increase in Dl^{EC} corresponds to a decrease in Dl^{FL} consistent with the notion that Dl^{FL} is the precursor of the Dl^{EC} product. In addition, these data indicate that transfection of Kuz acts additively to the endogenous Kuz in the S2 cells. Supporting this hypothesis, cotransfection 10 with KuzDN has a dramatic inhibitory effect on Dl^{EC} production (Figure 8A). Under identical experimental conditions cotransfection of Kuz or KuzDN has no effect on the proteolytic processing of Notch (Figure 8B). These observations demonstrate that Kuz plays a prominent role in the processing of Delta, one that is not as clear in the 15 processing of Notch. In agreement with this conclusion, it has been found Dl^{EC} production was markedly inhibited by the metalloprotease inhibitors EDTA and 1,10-phenanthroline (Figure 8C), while no effect was observed with serine protease inhibitors (PMSF and aprotinin), cysteine protease 20 inhibitor (leupeptin) or aspartyl protease inhibitor (pepstatin).

With the Dl^{EC} product showing to be present in embryos (Figure 7B), we sought to examine the role of Kuz in generating this product *in vivo*. kuz maternal null embryos 25 with either one (kuz +/-) or no (kuz -/-) zygotic copies of kuz were created by crossing female flies carrying kuz germline clones with kuz +/- male flies (Rooke et al., 1996, Nature 273:1227-1231). kuz -/- embryos were clearly distinguished from kuz +/- embryos by the absence of malpighian tubules and lack of movement. Extracts prepared 30 from a collection of nine of each type of embryo show the distinct absence of the Dl^{EC} and higher levels of Dl^{FL} in the Kuz -/- embryos as compared to Kuz +/- (Figure 8D). Re-

probing the same membrane with anti-Notch antibody showed no difference in processing of Notch in the *Kuz* $^{+/-}$ and *Kuz* $^{-/-}$ embryos. Furthermore, analysis of 14 randomly selected individual embryos showed eight embryos having significantly 5 higher levels of Dl^{FL} , analogous to the *kuz* $^{-/-}$ embryos (Figure 8D) and consistent with the predicted outcome of the cross. These observations indicate that *Kuz* mediated the proteolytic processing of Delta *in vivo*.

Although *kuz* mutations have multiple defects 10 indicating an involvement in different processes (Rooke et al., 1996, *Nature* 273:1227-1231), its phenotype partially overlaps with that of Delta. Inactivation of *kuz* during embryogenesis causes a more extensive neurogenic phenotype than Delta mutations, nevertheless, it is clear that in the ventrolateral region the neural hypertrophy in the two 15 mutations is identical. In adult mosaic clones, a small percentage of *kuz* mutant cells on the clone border develop into multiple bristles (Rooke et al., 1996, *Nature* 273:1227-1231). Delta mosaic clones present a more complicated situation. While cells on the border of the clones mutant 20 for weak delta alleles commit to epidermal fate, it is evident that cells mutant for strong delta alleles will develop multiple bristles at a low frequency (Figure 3 in Heitzler and Simpson, 1991, *Cell*, 1083-1092), the phenotype observed in *kuz* mutants. It is clear, however, that with 25 strong *kuz* and delta alleles, all extra neurons derive from genotypically mutant cells.

The above observations are distinct from a second function of *kuz* which has been termed neural promotion function (Rooke et al., 1996, *Science* 273:1227-1231; Rooke and Xu, 1998, *Bioassays* 20:209-214). This function prevents 30 cells in the center of *kuz* clones to develop bristles in contrast to the multiple bristle phenotype of delta clones. The genetic data, including the mosaic analyses, are

compatible with the hypothesis that the processing of Delta protein is mediated by Kuz. These findings are also compatible with earlier genetic studies linking kuz with Notch activity (Pan and Rubin, 1997, Cell 90:271-280; 5 Sotillos et al., 1997, Development 124:4769-4779; Wen et al., 1997, Development 124:4759-4767).

Adhesion assays have demonstrated that Notch-Delta interactions are physically mediated by the extracellular domains of the respective proteins (Fehon, et al., 1990, Cell 61:523-534). Furthermore deletion analyses have defined 10 specific sequences that are responsible for this interaction (Rebay, et al., 1991, Cell 67:687-699; deletion mutants of Delta lacking the DSL domain fail to bind Notch (M. Muskavitch, personal communication); Fleming et al., 1997, Development 124:2973-2981). These assays were done under 15 conditions where the Delta and the Notch proteins are overexpressed in S2 cells and full length Delta is clearly detected on the cell surface (data not shown). Interest was expressed to examine if Dl^{EC} is capable of binding to Notch.

Addition of Dl^{EC} to Notch expressing S2 cells 20 followed by sedimentation through a sucrose cushion resulted in specific binding of Dl^{EC} to the Notch cells as compared to S2 cells alone (Figure 9A) suggesting a Dl^{EC}:Notch complex forms on these cells. These results were extended by analyzing the ability of Dl^{EC} to compete for full length Delta 25 binding to Notch in a cell aggregation assay. In order to quantify the Notch/Delta interactions we have developed a turbidimetric assay which allows us to measure aggregation in a reproducible manner. Expression of Notch and Delta in S2 cells are induced for 16 hours with 0.085 mM and 0.022 mM CuSO₄, respectively. The cells are then centrifuged and 30 raised in serum free media to an equivalent density yielding between 20-30% T_{320nm} (~2x10⁶ cells/mL) in a Benchtop spectrophotometer. Blank values are set with M3 media alone.

400 μ L of Notch and 400 μ L of Delta cells are then pipeted into a 1.4 mL black sided, stoppered quartz cuvette and quickly inverted three times. The $T_{320\text{nm}}$ is read immediately to determine the time "zero" value. The cuvette is then 5 rocked horizontally on a Thermolyne vari-mixer at 20 oscillations per minute and subsequent $T_{320\text{nm}}$ readings are taken at one minute intervals. Change in $T_{320\text{nm}}$ (relative to time zero) is then plotted versus time. The effect of Dl^{EC} was compared to a concentrate of media from $\Delta\text{ECN-S2}$ cells 10 (closed squares) (Rebay et al., 1993, Cell 74:318-329) prepared in parallel as these cells were transfected in the same manner with an irrelevant construct.

Pre-incubation of the Notch cells with Dl^{EC} concentrate resulted in a dramatic reduction in the initial rate of aggregation with Delta cells (Figure 9B). The 15 competitive effect of Dl^{EC} was sensitive to the concentration added and the time of preincubation with the Notch cells. Furthermore, pre-incubation of the Delta cells with Dl^{EC} had no effect on subsequent aggregation with Notch cells indicating Dl^{EC} specifically binds to Notch in a competitive 20 manner with respect to full length Delta.

The biological activity of Dl^{EC} was examined in a cell culture assay which was carried out as follows. Low density primary cultures of cortical neurons were prepared from embryonic day 15.5 to 16.5 mouse embryos. Single cell 25 suspensions in Dulbecco's modified Eagle medium high glucose/F12 (1:1), N2 Supplement, 2.5 mM L-glutamine and 5-10% fetal bovine serum were seeded on 5 mm diameter glass coverslips precoated with 15 μ g/ml poly-ornithine and 2 μ g/cm² laminin. After 10 days in culture, neurons (<1000/cm²) were growing on a monolayer of glial cells. To examine the 30 activity of Dl^{EC} , cultures were treated for 14-17 hours with a 1:10 dilution of either 5X ΔECN , 5X Dl^{EC} , purified Dl^{EC} (approximately 0.04 $A_{280\text{nm}}$ /ml in 25 mM glycine, 30 mM Tris-HCl,

pH 7.0) made in culture medium. At least three independent culture wells were examined for each condition during one experimental trial. Cells were fixed in 4% paraformaldehyde, stained overnight with a mouse monoclonal antibody against 5 neuron-specific class III β -tubulin (TuJ1, 1:500; BabCo, Berkeley, CA) and visualized with Cy3 conjugated anti-mouse secondary antibody (Jackson Immunoresearch Laboratories). Immunolabeled neurons were imaged with a Spot2 camera (Diagnostic Instruments) using a 40X objective on a Zeiss 10 Axioplan 2 microscope and imported into Adobe Photoshop 4.0 (Adobe Systems, San Jose, CA). Neurite length was measured in five to ten randomly selected images from each coverslip using NIH Image 1.61 software and the data were analyzed with Sigma Plot 4.0 statistical software (SPSS).

Primary cultures of mouse cortical neurons 15 expressing Notch endogenously develop dendritic processes (Figure 9C). It has been demonstrated that ligand-dependent Notch activation *in vitro* in cortical neurons expressing endogenous Notch receptors causes morphological changes and retraction of neurites. The same effects were observed when 20 the neurons were cultured in the presence of enriched Dl^{EC} containing media or purified Dl^{EC} (Figure 9C). These data show that Dl^{EC} has biological activity consistent with the notion that it acts as an agonist. Similar effects of neurite outgrowth have also been observed with a soluble form 25 of vertebrate Jagged (unpublished observation).

Amino acid sequence analysis was performed on the soluble Delta peptide. As described above, the molecular weight of Dl^{EC} estimated from SDS-PAGE analysis is consistent with Dl^{EC} being comprised of most if not all of the extracellular portion of the Delta protein. In addition, the 30 N-terminal sequence of Dl^{EC} is consistent with the predicted N-terminus of full length Delta (Dl^{EC} is not proteolytically clipped at the N-terminus). Further, as described above, Dl^{EC}

likely arises due to proteolytic processing at a cleavage site(s) between the ninth EGF repeat and the transmembrane domain in a region designated the juxtamembrane domain. The sequence analysis was carried out by C-terminal sequencing 5 and by tryptic digest/liquid chromatography/mass spectrometry (LC/MS) of purified Dl^{EC} derived from *Drosophila* Delta expressed in S2 cells. This analysis was carried out at the Harvard Microchemistry Facility, Cambridge MA.

The data generated by the C-terminal sequencing 10 showed that the terminal residue was alanine. The amino acid residue preceding the terminal residue showed heterogeneity with glycine being the most prevalent followed by asparagine, leucine, and arginine. These data indicate that Dl^{EC} terminates at more than one position which indicates that more than one proteolytic processing site exists. However, 15 C-terminal sequencing is very difficult to perform and the confidence of residues beyond the terminal residue drops off significantly. However, analysis of the *Drosophila* Delta juxtamembrane domain (residues 564-594 of *Drosophila* Delta) shows four of six possible alanine residues that would give a 20 terminal sequence consistent with the C-terminal sequencing data, i.e., DA₅₇₆, GA₅₈₁, LA₅₉₁, and NA₅₉₃, (Figure 11). Our data indicated an alanine at position 591, in contrast to the sequence data of Vässin, et al.; 1987, EMBO J. 6:3431-3440, which disclosed a threonine at that position.

25 The tryptic digest peptide analysis was consistent with the C-terminal sequencing data. 24 tryptic digest peptides derived from *Drosophila* Dl^{EC} were positively identified by LC/MS and their sequences determined. Five peptides were identified that terminated in the juxtamembrane domain. Two of the peptides terminated at residue Ala₅₉₃ and 30 two other peptides terminated at residue Ala₅₈₁. These data demonstrate that two prevalent forms of Dl^{EC} terminate at amino acid residues 581 and 593. The fifth peptide

terminated at amino acid position Gln_{578} , which was not detected in the C-terminal analysis. The resolution of Ala_{581} and Ala_{593} by both analytical methods together indicates that the primary forms of Dl^{EC} are generated by cleavage at these 5 sites, although additional cleavage sites remain a possibility. The nature of these analyses do not permit a quantitative assessment of the relative proportion of the various species, thus it cannot be concluded which of the cleavage sites are preferred.

10 In conclusion, genetic and biochemical data show that Delta is cleaved to produce an active, functionally important extracellular fragment that is biologically active with an apparent agonistic function in the Notch pathway. Previous studies involving the *in vivo* expression of artificially truncated Notch ligands in *Drosophila* and other 15 systems have demonstrated both antagonistic and agonistic activities (Sun et al., 1997, *Development* 124:3439-3448; Fitzgerald et al., 1995, *Development* 121:4275-4282; Li et al., 1998, *Immunity* 8:43-55; Wang et al., 1998, *Neuron* 21:63-75). It is clear that soluble forms of Delta (Dls) can act 20 as antagonists in the developing *Drosophila* eye (Sun et al., 1997, *Development* 124:3439-3448). However, Dl^{EC} is not identical to Dls and therefore it is plausible that the two molecules may be functionally different. Figure 10 is a schematic comparing Dl^{EC} and Dls.

25 Although Kuz does not appear to be responsible for the constitutive cleavage of Notch, the possibility that Kuz can cleave Notch at alternative sites remains. In this regard, it has been claimed that KuzDN is able to inhibit transactivation of a target gene of the Notch pathway induced by ligand binding to the receptor (Logeat, et al., 1998, 30 *Proc. Natl. Acad. Sci. USA* 95:8108-8112). However it is possible that this effect does not reflect Notch cleavage but rather the cleavage of Delta to produce an active ligand.

Klueg et al., 1998, Mol. Cell Biol. 9:1709-1723 ("Klueg") have recently reported the processing of Delta during normal embryogenesis demonstrating the existence of Delta fragments, one of which is consistent with Dl^{EC} . We note the 5 intermediate forms detected in the 16-20 hour embryos (Figures 7B, 8D, *kuz* +/-) are not present in *Kuz* mutants (Figure 8D, *kuz* -/-), raising the possibility that the generation of these products may also be mediated by *Kuz*.

The present invention is not to be limited in scope 10 by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

15 Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

1. A peptide comprising a fragment of a Delta protein, the amino acid sequence of the peptide consisting of the amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).
10 2. The peptide of claim 1 which comprises 30 contiguous amino acids of a Delta protein.
3. The peptide of claim 1 which comprises 100 contiguous amino acids of a Delta protein.
15 4. The peptide of claim 1 which comprises 150 contiguous amino acids of a Delta protein.
5. A purified derivative or analog of the peptide of claim 1, which is able to display one or more functional activities of a Delta cleavage peptide.
20 25 6. A purified derivative or analog of the peptide of claim 1, which is able to display one or more functional activities of a human or *D. melanogaster* Delta cleavage peptide.
30 7. The derivative or analog of claim 5 which is able to be bound by an antibody directed against a human or *D. melanogaster* Delta cleavage peptide.

8. A purified fragment of the peptide of claim 1, which is able to be bound by an antibody directed against a human Delta cleavage peptide.

5 9. A molecule comprising the fragment of claim 8.

10 10. A purified fragment of the peptide of claim 1 which is able to display one or more functional activities of a human Delta cleavage peptide.

10 11. A chimeric protein comprising a fragment of a Delta protein of not more than 200 amino acids, said fragment comprising the amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick
15 Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9), fused via a covalent bond to an amino acid sequence of a second protein, in which the second protein is not the fragment of the Delta protein.

20 12. The chimeric protein of claim 11 in which the Delta cleavage peptide is of a human protein.

25 13. The chimeric protein of claim 12 which is able to display one or more functional activities of a Delta cleavage peptide.

14. The peptide of claim 1 which is purified.

30 15. A fragment of a Delta protein of not more than 150 amino acids comprising a Delta sequence selected from the group consisting of amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃

in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), and Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

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16. A fragment of a Delta protein of not more than 50 amino acids comprising a Delta sequence selected from the group consisting of amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), and Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

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17. A fragment of a Delta protein of not more than 30 amino acids comprising a Delta sequence selected from the group consisting of amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), and Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

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18. A peptide the amino acid sequence consisting of amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

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19. A molecule comprising the fragment of claim 15, 16 or 17 or the peptide of claim 18.

20. A chimeric protein comprising a Delta protein sequence fused to a non-Delta protein sequence, wherein the Delta protein sequence is a sequence of not more than 100 amino acids that comprises the amino acid sequence Cys₅₁₆ to 5 amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

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21. A chimeric protein comprising a Delta protein sequence fused to a non-Delta protein sequence, wherein the Delta protein sequence is a sequence of not more than 50 amino acids that comprises the amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to 15 amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

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22. A chimeric protein comprising a Delta protein sequence fused to a non-Delta protein sequence, wherein the Delta protein sequence is a sequence of not more than 30 amino acids that comprises the amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to 25 amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

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23. A chimeric protein comprising a Delta protein sequence fused to a non-Delta protein sequence wherein the Delta sequence is selected from the group consisting of amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ

ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), and Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ 5 ID NO:9).

24. The fragment or peptide of claim 15, 16, 17 or 18, which is purified.

10 25. An antibody, or a fragment thereof, directed against the Delta sequence of the fragment or peptide of claim 15, 16, 17 or 18 or the chimeric protein of claim 20, 21 or 22.

15 26. A method of modulating activity of Notch or Delta or Kuz or at least one of their signalling pathways in a cell, or organism comprising a cell, that expresses Notch or Delta or Kuz comprising contacting the cell or organism with the fragment of claim 15, 16 or 17 or peptide or molecule of claim 18 or 19 or protein of claim 20, 21 or 22 20 or the fragment or peptide of claim 24.

25 27. A method of modulating activity of Notch or Delta or Kuz or at least one of their signalling pathways in a cell or organism that expresses Notch or Delta or Kuz comprising contacting the cell or organism with the antibody of claim 25.

30 28. A method of modulating activity of Notch or Delta or Kuz or at least one of their signalling pathways in a cell or organism that expresses Notch or Delta or Kuz comprising recombinantly expressing within the cell or organism the fragment of claim 15, 16 or 17 or peptide or

molecule of claim 18 or 19 or protein of claim 20, 21 or 22 or the fragment or peptide of claim 24.

29. A method of modulating activity of Notch or
5 Delta or Kuz or at least one of their signalling pathways in a cell or organism that expresses Notch or Delta or Kuz comprising recombinantly expressing within the cell or organism the antibody of claim 25.

10 30. The fragment of claim 15, 16 or 17 or the peptide of claim 18, which is amino- or carboxy-terminal derivatized.

15 31. The fragment or peptide of claim 30 which is N-acetylated.

32. The fragment or peptide of claim 30 which has a C-terminal amide.

33. A kit comprising in a container the fragment 20 of claim 15, 16 or 17 or the peptide of claim 18.

34. A pharmaceutical composition comprising the fragment of claim 15, 16 or 17 or the peptide of claim 18, in purified form; and a pharmaceutically acceptable carrier.

25 35. A transgenic non-human animal containing a transgene encoding the fragment of claim 15, 16 or 17 or the peptide of claim 18 or the protein of claim 20, 21 or 22.

36. A nucleic acid comprising a nucleotide 30 sequence encoding a fragment of Delta of not more than 200 amino acids, said fragment comprising the amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅

to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

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37. The nucleic acid of claim 36 which is isolated.

38. The nucleic acid of claim 36 which is DNA.

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39. An isolated nucleic acid comprising a nucleotide sequence complementary to the nucleotide sequence of claim 36.

40. A recombinant cell containing the nucleic acid 15 of claim 36.

41. A method of producing a Delta cleavage peptide comprising growing a recombinant cell containing the nucleic acid of claim 36 such that the encoded Delta cleavage peptide 20 is expressed by the cell, and recovering the expressed Delta cleavage peptide.

42. The product of the process of claim 41.

25 43. A pharmaceutical composition comprising a therapeutically effective amount of a fragment of a Delta protein, the amino acid sequence of the fragment consisting of the amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta 30 (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila*

Delta (SEQ ID NO:9); and a pharmaceutically acceptable carrier.

44. The composition of claim 43 in which the Delta 5 protein is a human Delta protein.

45. A pharmaceutical composition comprising a therapeutically effective amount of a derivative or analog of a Delta cleavage peptide, which derivative or analog is characterized by the ability to bind to a Kuz protein; and a 10 pharmaceutically acceptable carrier.

46. A pharmaceutical composition comprising a therapeutically effective amount of the nucleic acid of claim 36; and a pharmaceutically acceptable carrier.

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47. A pharmaceutical composition comprising a therapeutically effective amount of an antibody which binds to a Delta cleavage peptide and a pharmaceutically acceptable carrier.

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48. A pharmaceutical composition comprising a therapeutically effective amount of a fragment or derivative of an antibody to a Delta cleavage peptide containing the binding domain of the antibody; and a pharmaceutically acceptable carrier.

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49. A method of treating or preventing a disease or disorder in a subject comprising administering to a subject in which such treatment or prevention is desired a therapeutically effective amount of a fragment of a Delta 30 protein, the amino acid sequence of the fragment consisting of the amino acid sequence Cys₅₁₆ to amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10), Cys₅₁₅ to amino acid Phe₅₄₃ in mouse

Delta (SEQ ID NO:6), Cys₅₂₃ to amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7), Cys₅₁₈ to amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8), or Cys₅₆₄ to amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9), or a derivative of any of the foregoing 5 which is able to bind to a Kuz protein.

50. The method according to claim 49 in which the disease or disorder is a malignancy characterized by increased Delta activity or increased expression of a Delta 10 protein or of a Delta derivative capable of being bound by an anti-Delta antibody, relative to said Delta activity or expression in an analogous non-malignant sample.

51. The method according to claim 50 in which the disease or disorder is selected from the group consisting of 15 cervical cancer, breast cancer, colon cancer, melanoma, seminoma, and lung cancer.

52. The method according to claim 50 in which the subject is a human.

20

53. A method of treating or preventing a disease or disorder in a subject comprising administering to a subject in which such treatment or prevention is desired an effective amount of the nucleic acid of claim 36.

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54. The method according to claim 50 in which the disease or disorder is a disease or disorder of the central nervous system.

55. A method of diagnosing a disease or disorder 30 characterized by an aberrant level of Notch-Delta protein binding activity in a patient, comprising measuring the ability of a Delta cleavage peptide in a sample derived from

the patient to bind to a Kuz protein, in which an increase or decrease in the ability of the peptide to bind to the Kuz protein, relative to the ability found in an analogous sample from a normal individual, indicates the presence of the 5 disease or disorder in the patient.

56. A purified complex of a Delta protein and a Kuz protein.

10 57. The purified complex of claim 56 in which the proteins are human proteins.

15 58. A purified complex selected from the group consisting of a complex of a derivative of Delta and Kuz, a complex of Delta and a derivative of Kuz, and a complex of a derivative of Delta and a derivative of Kuz; in which the derivative of Delta is able to form a complex with a wild-type Kuz and the derivative of Kuz is able to form a complex with wild-type Delta.

20 59. The purified complex of claim 58 in which the derivative of Delta or Kuz is fluorescently labeled.

25 60. A chimeric protein comprising a fragment of Delta consisting of at least 6 amino acids fused via a covalent bond to a fragment of Kuz consisting of at least 6 amino acids.

30 61. The chimeric protein of claim 60 in which the fragment of Delta is a fragment capable of binding Kuz and in which the fragment of Kuz is a fragment capable of binding Delta.

62. The chimeric protein of claim 61 in which the fragment of Delta and the fragment of Kuz form a Delta:Kuz complex.

5 63. An antibody which immunospecifically binds the complex of claim 58 or a fragment or derivative of said antibody containing the binding domain thereof.

10 64. The antibody of claim 63 which does not immunospecifically bind Delta or Kuz that is not part of a Delta:Kuz complex.

15 65. An isolated nucleic acid or an isolated combination of nucleic acids comprising a nucleotide sequence encoding a Delta protein and a nucleotide sequence encoding a Kuz protein.

20 66. The isolated nucleic acid or isolated combination of nucleic acids of claim 65 which are nucleic acid vectors.

25 67. The isolated nucleic acid or isolated combination of nucleic acids of claim 65 in which the Delta coding sequence and the Kuz coding sequence are operably linked to a promoter.

68. An isolated nucleic acid that comprises a nucleotide sequence encoding the chimeric protein of claim 60.

30 69. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the complex of claim 56; and a pharmaceutically acceptable carrier.

70. The pharmaceutical composition of claim 70 in which the proteins are human proteins.

71. A method of producing a complex of Delta and 5 Kuz comprising growing a recombinant cell containing the nucleic acid of claim 65 such that the encoded Delta and Kuz proteins are expressed and bind to each other, and recovering the expressed complex of Delta and Kuz.

10 72. A method of diagnosing or screening for the presence of or a predisposition for developing a disease or disorder characterized by an aberrant level of a complex of Delta and Kuz, in a subject comprising measuring the level of said complex, RNA encoding Delta and Kuz, or functional activity of said complex in a sample derived from the 15 subject, in which an increase or decrease in the level of said complex, said RNA encoding Delta and Kuz, or functional activity of said complex in the sample, relative to the level of said complex, said RNA encoding Delta and Kuz or functional activity of said complex found in an analogous 20 sample not having the disease or disorder or a predisposition for developing the disease or disorder, indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

25 73. A kit comprising in one or more containers a substance selected from the group consisting of a complex of Delta and Kuz, an antibody against said complex, nucleic acid probes capable of hybridizing to RNA of Delta and RNA of Kuz, or pairs of nucleic acid primers capable of priming amplification of at least a portion of the Delta gene and the 30 Kuz gene.

74. A method of treating or preventing a disease or disorder involving aberrant levels of a complex of Delta and Kuz, in a subject comprising administering to a subject in which such treatment or prevention is desired a 5 therapeutically effective amount of a molecule or molecules that modulate the function of said complex.

75. A method of screening for a molecule that 10 modulates directly or indirectly the formation of a complex of Delta and Kuz comprising measuring the levels of said complex formed from Delta and Kuz proteins in the presence of said molecule under conditions conducive to formation of the complex; and comparing the levels of said complex with the levels of said complex that are formed in the absence of said 15 molecule, wherein a lower or higher level of said complex in the presence of said molecule indicates that the molecule modulates formation of said complex.

76. A recombinant non-human animal in which both 20 an endogenous *Delta* gene and an endogenous *Kuz* have been deleted or inactivated by homologous recombination or insertional mutagenesis of said animal or an ancestor thereof.

25 77. A recombinant non-human animal containing both a *Delta* gene and a *Kuz* gene, in which the *Delta* gene is under the control of a promoter that is not the native *Delta* gene promoter and the *Kuz* gene is under the control of a promoter that is not the native *Kuz* gene promoter.

30 78. A method of modulating the activity or levels of Delta by contacting a cell with, or administering an animal expressing a *Delta* gene, a *Kuz* protein, or a nucleic

acid encoding said protein or an antibody that immunospecifically binds said protein or a fragment or derivative of said antibody containing the binding domain thereof.

5

79. A method of modulating the activity or levels of Kuz by contacting a cell with, or administering an animal expressing a gene encoding said protein, Delta, or a nucleic acid encoding Delta, or an antibody that immunospecifically binds Delta or a fragment or derivative of said antibody containing the binding domain thereof.

10

80. A method for identifying a molecule that modulates activity of Delta or Kuz or a complex of Delta and Kuz comprising contacting one or more candidate molecules with Delta in the presence of Kuz; and measuring the amount of complex that forms between Delta and Kuz; wherein an increase or decrease in the amount of complex that forms relative to the amount that forms in the absence of the candidate molecules indicates that the molecules modulate the activity of Delta or Kuz or said complex of Delta and Kuz.

15

81. A method for detecting or measuring Delta activation in a cell comprising detecting or measuring the expression of one or more Delta cleavage products selected from the group consisting of Dl^{EC} and Dl^{TM} , wherein the presence and amount of Delta cleavage products indicates the presence and amount, respectively, of Delta activation.

25

82. The method according to claim 81 in which said detecting or measuring is carried out by a method comprising contacting a cell with a molecule that binds to Dl^{EC} or Dl^{TM} under conditions conducive to specific binding; and detecting any binding of the molecule to the cell that occurs.

83. The method according to claim 82 in which the molecule is an anti-Delta antibody or a binding region thereof.

5 84. The method according to claim 82 in which the molecule is Notch or Kuz or a binding region thereof.

10 85. The method according to claim 83 in which the antibody or binding region thereof is labelled with a fluorescent label, and binding of the antibody to the cell is detected or measured by fluorescent activated cell sorting.

15 86. The method according to claim 81 in which said detecting or measuring is carried out by a method comprising (a) contacting the cell with a reagent that binds to or reacts with cell surface proteins under conditions conducive to such binding or reaction; and (b) detecting any such binding to or reaction with Delta.

20 87. The method according to claim 86 in which the reagent is labeled.

25 88. The method according to claim 86 in which said detecting is carried out by a method comprising contacting the cell with a labeled specific binding partner to the reagent.

30 89. The method according to claim 87 or 88 in which the detecting of any such binding or reaction in step (b) is carried out by western blotting or immunoprecipitation, using an anti-Delta antibody.

90. A method for detecting or measuring Delta activation in a cell comprising detecting or measuring an

amino-terminal fragment of Delta terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ in *Drosophila* Delta, between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta, between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta, between 5 amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta, or terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta.

91. A method for detecting or measuring Delta activation in a cell comprising detecting or measuring under 10 reducing conditions, a soluble Delta fragment of approximately 67 kilodaltons.

92. A method for detecting or measuring Kuz function in a cell comprising detecting or measuring the 15 expression of one or more Delta cleavage products selected from the group consisting of Dl^{EC} and DlTM, wherein the presence and amount of Delta cleavage products indicates the presence and amount, respectively, of Kuz function.

93. The method according to claim 92 in which said detecting or measuring is carried out by a method comprising contacting a cell with a molecule that binds to Dl^{EC} or DlTM under conditions conducive to specific binding; and detecting any binding of the molecule to the cell that occurs.

94. The method according to claim 93 in which the 25 molecule is an anti-Delta antibody or a binding region thereof.

95. A method for detecting or measuring Kuz 30 function in a cell comprising detecting or measuring an amino-terminal fragment of a Delta protein terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ in *Drosophila*

Delta, between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta, between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta, between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta, or terminating between amino acid Cys₅₁₈ and amino acid 5 Phe₅₄₄ in *Xenopus* Delta.

96. A method for detecting or measuring Kuz function in a cell comprising detecting or measuring under reducing conditions, a soluble Delta fragment of approximately 67 kilodaltons.
10

97. A method for identifying a modulator of Delta activation comprising providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more Delta cleavage products selected 15 from the group consisting of Dl^{EC} and DlTM, in which a difference in the presence or amount of said one or more cleavage products compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Delta activity.
20

98. A method for identifying a modulator of Delta activation comprising contacting a cell with a candidate modulator molecule and detecting or measuring the amount of the expression an amino-terminal fragment of a Delta protein terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ in 25 *Drosophila* Delta, between amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta, between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta, between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta, or terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta.; in which a difference 30 in the presence or amount of said fragment compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

99. A method for identifying a modulator of Delta activation comprising contacting a cell with a candidate modulator molecule and detecting or measuring under reducing conditions, a soluble Delta fragment of approximately 67 kilodaltons, in which a difference in the presence or amount of said soluble Delta fragment compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

100. A method for identifying a modulator of Kuz function comprising providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more Delta cleavage products selected from the group consisting of Dl^{EC} and Dl^{TM} , in which a difference in the presence or amount of said one or more 15 cleavage products compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Kuz function.

101. A method for identifying a modulator of Kuz function comprising contacting a cell with a candidate modulator molecule and detecting or measuring the amount of the expression an amino-terminal fragment of a Delta protein terminating between amino acid Cys_{564} and amino acid Ala_{593} in *Drosophila* Delta, between amino acid Cys_{516} and amino acid Phe_{543} in human Delta, between amino acid Cys_{515} and amino acid Phe_{543} in mouse Delta, between amino acid Cys_{523} and amino acid Phe_{551} in chick Delta, or terminating between amino acid Cys_{518} and amino acid Phe_{544} in *Xenopus* Delta.; in which a difference 25 in the presence or amount of said fragment compared to a Delta cell not contacted with the candidate molecule 30 indicates that the molecule modulates Kuz function.

102. A method for identifying a modulator of Kuz function comprising contacting a cell with a candidate modulator molecule and detecting or measuring under reducing conditions, a soluble Delta fragment of approximately 67.5 kilodaltons, in which a difference in the presence or amount of said soluble Delta fragment compared to a Delta cell not contacted with the candidate molecule indicates that the molecule modulates Kuz function.

103. A method for identifying a modulator of Delta activation comprising contacting a candidate modulator molecule with a full length Delta in the presence of Kuz and optionally a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of the composition and detecting or measuring the amount of Delta cleavage products $D1^{EC}$ and $D1^{TM}$ that result, in which a difference in the presence or amount of said Delta cleavage products compared to a full-length Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Delta activity.

104. The method according to claim 103 in which the composition is a cell lysate made from cells which recombinantly express Delta.

105. The method according to claim 103 in which the composition is a cell lysate made from cells which endogenously express Delta.

106. A method for identifying a modulator of Kuz function comprising contacting a candidate modulator molecule with a full length Delta in the presence of Kuz and optionally a composition comprising cellular proteins, under

conditions conducive to cleavage of the full-length Delta by Kuz and optionally one or more components of the composition and detecting or measuring the amount of Delta cleavage products D₁^{EC} and D₁TM that result, in which a difference in 5 the presence or amount of said Delta cleavage products compared to a full-length Delta in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Kuz function.

107. The method according to claim 106 in which 10 the composition is a cell lysate made from cells which recombinantly express Kuz.

108. The method according to claim 106 in which the composition is a cell lysate made from cells which 15 endogenously express Kuz.

109. A purified fragment of a Delta protein, the sequence of said fragment consisting of the amino acid sequence beginning at amino acid Ser₂₂ and terminating between 20 amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7); beginning at amino acid Ser₂₂ and terminating between 25 amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); and the sequence beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

110. The fragment of claim 109, which is amino- or carboxy-terminal derivatized.

111. The fragment of claim 110, which is N-acetylated.

112. The fragment of claim 110, which has a C-5 terminal amide.

113. A nucleic acid comprising a nucleotide sequence encoding a fragment of a Delta protein, the amino acid sequence of said fragment consisting of an amino acid sequence beginning at amino acid Ser₂₂ and terminating between 10 amino acid Cys₅₁₆ and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID 15 NO:7); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); and the sequence beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9).

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114. The nucleic acid of claim 113 which is isolated.

25 115. The nucleic acid of claim 113 which is DNA.

116. An isolated nucleic acid comprising a nucleotide sequence complementary to the nucleotide sequence encoding a fragment of Delta of nucleic acid of claim 113.

30 117. A peptide comprising a fragment of a Delta protein, the amino acid sequence of the fragment consisting of the amino acid sequence Cys₅₆₄ to amino acid Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9).

118. A chimeric protein comprising a fragment of a Delta protein of not more than 200 amino acids, said fragment comprising the amino acid sequence Cys₅₆₄ to amino acid Gln₅₉₄ in *Drosophila Delta* (SEQ ID NO:9), fused via a covalent bond 5 to an amino acid sequence of a second protein, in which the second protein is not the fragment of the Delta protein.

119. A peptide the amino acid sequence of which consists of amino acid sequence Cys₅₆₄ to amino acid Gln₅₉₄ in 10 *Drosophila Delta* (SEQ ID NO:9).

120. A nucleic acid comprising a nucleotide sequence encoding a fragment of a Delta protein of not more than 200 amino acids, said fragment comprising the amino acid sequence Cys₅₆₄ to amino acid Gln₅₉₄ in *Drosophila Delta* (SEQ 15 ID NO:9).

121. A method for detecting or measuring Delta activation in a cell comprising detecting or measuring an amino-terminal fragment of Delta terminating between amino 20 acid Cys₅₆₄ and amino acid Gln₅₉₄ in *Drosophila Delta*.

122. A method for detecting or measuring Kuz function in a cell comprising detecting or measuring an amino-terminal fragment of a Delta protein terminating 25 between amino acid Cys₅₆₄ and amino acid Gln₅₉₄ in *Drosophila Delta*.

123. A purified fragment of a Delta protein, the amino acid sequence of said fragment consisting of the amino acid sequence beginning at amino acid Ser₂₃ and terminating 30 between amino acid Cys₅₆₄ and amino acid Gln₅₉₄ in *Drosophila Delta* (SEQ ID NO:9).

124. A nucleic acid comprising a nucleotide sequence encoding a fragment of a Delta protein, the amino acid sequence of said fragment consisting of an amino acid sequence beginning at amino acid Ser₂₃ and terminating between 5 amino acid Cys₅₆₄ and amino acid Gln₅₉₄ in *Drosophila Delta* (SEQ ID NO:9).

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AMENDED CLAIMS

[received by the International Bureau on 17 March 2000 (17.03.00);
new claims 125 and 126 added; remaining claims unchanged (1 page)]

124. A nucleic acid comprising a nucleotide sequence encoding a fragment of a Delta protein, the amino acid sequence of said fragment consisting of an amino acid sequence beginning at amino acid Ser₂₃ and terminating between 5 amino acid Cys₅₆₄ and amino acid Gln₅₉₄ in *Drosophila* Delta (SEQ ID NO:9).

125. A fragment of the soluble Delta peptide, which peptide consists of the amino acid sequence beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₆ 10 and amino acid Phe₅₄₃ in human Delta (SEQ ID NO:10); beginning at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₅ and amino acid Phe₅₄₃ in mouse Delta (SEQ ID NO:6); beginning at amino acid Ser₂₄ and terminating between amino acid Cys₅₂₃ and amino acid Phe₅₅₁ in chick Delta (SEQ ID NO:7); beginning 15 at amino acid Ser₂₂ and terminating between amino acid Cys₅₁₈ and amino acid Phe₅₄₄ in *Xenopus* Delta (SEQ ID NO:8); and the sequence beginning at amino acid Ser₂₃ and terminating between amino acid Cys₅₆₄ and amino acid Ala₅₉₃ in *Drosophila* Delta (SEQ ID NO:9), which fragment is able to bind a Notch 20 protein.

126. An isolated soluble Delta peptide isolated by a process comprising:

(a) culturing a Delta-expressing cell in cell culture medium, such that the cell secretes a soluble Delta peptide into the cell culture medium; and 25
(b) isolating the soluble Delta peptide from the cell culture medium.

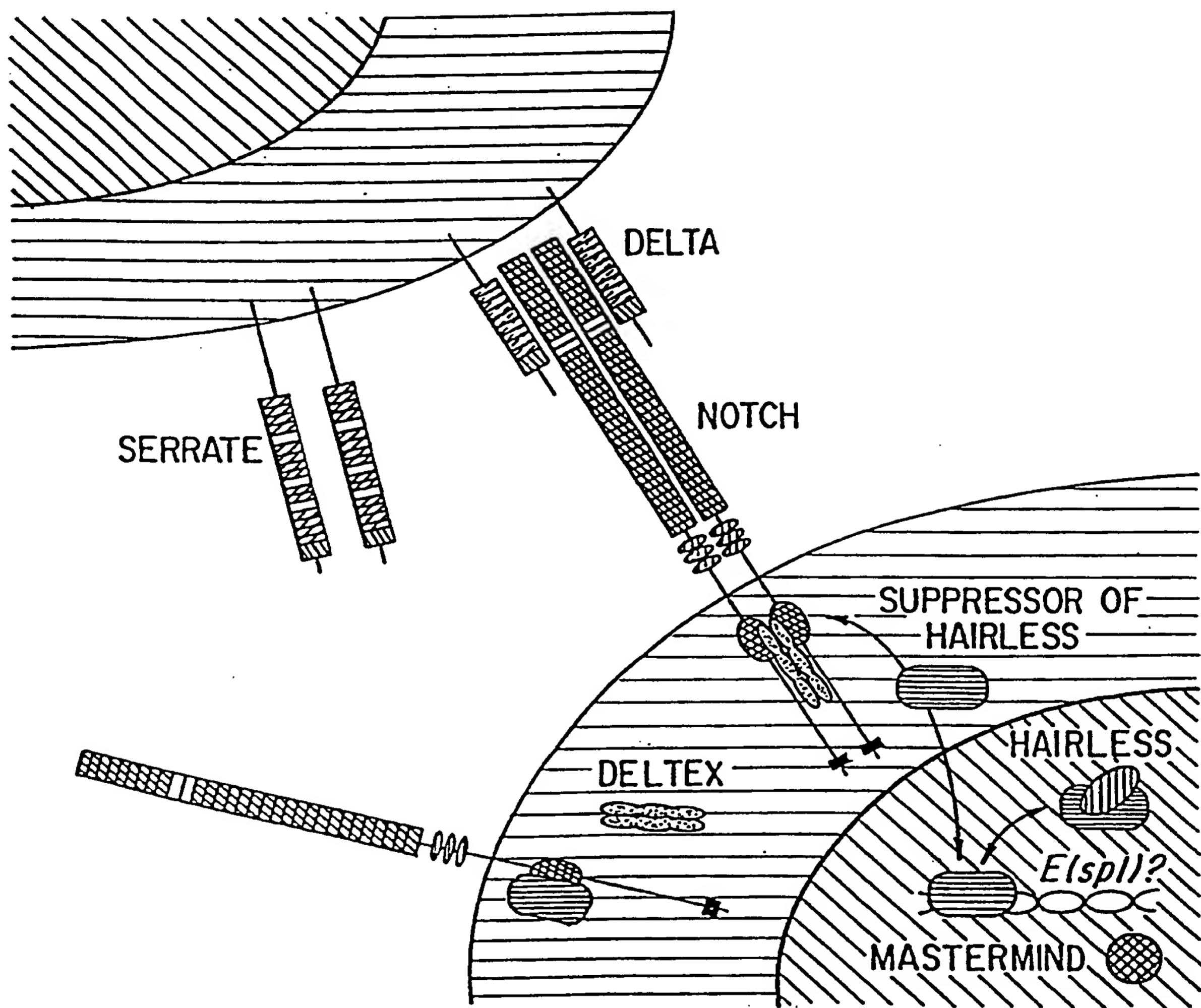


FIGURE 1

Alignment of Notch Homologs

Potential Input Cloning Set		EGF Reports	
Ime0	-----	-----	111
Ime1	-----	-----	159
Ime2	-----	-----	169
Ime3	-----	-----	244
Ime4	-----	-----	259
Ime5	-----	-----	265
Ime6	-----	-----	265
Ime7	-----	-----	292
Ime8	-----	-----	408
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Ime218	-----	-----	6

FIGURE 3

588 D C - - A T N A Q - - - Y V L I A V F S V A M P L V - - - A V I A A L V V F C M K R K R K R A G D S L
 589 E K Y - - T E C G S N O F P V I A V C A C I I V L V L L L G C A A V V V C V - - - - - R V R V G X D L
 590 E K Y - - T E C G O N S D F P V I A V C A G I I V L V L L L G C A A V V V C V - - - - - R L K V O C D L
 591 E R - H M E S O C G P F P U V A V C A G V V L V L L L G C A A V V V C V - - - - - P L K L C M D L
 592 E R - H M E S O C G P F P U V A V C A G V V L V L L L G C A A V V V C V - - - - - P L K L C M D L
 593 E R - H M E S O C G P F P U V A V C A G V V L V L L L G C A A V V V C V - - - - - P L K L C M D L
 594 E R - H M E S O C G P F P U V A V C A G V V L V L L L G C A A V V V C V - - - - - P L K L C M D L
 TM

628 E K D D A E A R K D N E D N A V A T P H M N G S G V G V A L A S A S L G G K T G S N S C L T F D G D S L
 576 K R R H O P E A C R G E S K I T M N N L A N C G R E X D I S Y S F I G T T O I K N T N K K I D F L S E X D L
 583 K R R H O P E A C R G E S K I T M N N L A N C G R E X D I S Y S F I G T T O I K N T N K K V D F H S E C D L
 575 K R R H O P P E P C W C E T T M N N L A N C G R E X D I S Y S I I G A T O I K N T N K K A D F H S D M D L

678 N P N I I K N T W O K S V N N I C A S A A A A A A A A A A A E C L H Y G C Y V A S Y A D I N N N A N D S L
 626 S N N E - - - - - K N G Y K P R Y P S V G Y N L V H E L K N E D - - - - - S P K E E R Y K C X D L
 633 - N S D - - - - - K N G Y K V R Y P T S V D Y N L V H E L K N E D - - - - - S V K E E R Y K C C D L
 625 H G A K - - - - - K S S P K V R Y P T V D Y N L V R C L K G C E - - - - - A T V R D T H S K X M D L
 625 H S A D - - - - - K N G F K A R Y F A V D Y N I Y D I K G C D - - - - - T A Y P D S I C K S M D L

728 S D F C V A P L O R A A S D K M L N T C P T L R M R C S P A G S S A K W A S G - G C F G A E G K R D S L
 662 E - - - - - A X T S S N - - - - - D D S E C V N S V H - - - S K R D S S S E R A X D L
 668 E - - - - - A K C E T Y - - - - - D S E A E E K S A V O - - - L K S S G T T C F R K C C D L
 662 O - - - - - K C O S C - - - - - S S A G E E X I A P T - - - L N G G E I P D I R K C P D L
 662 D - - - - - T K F C P D - - - - - G S S I F Y I T P T T R G G F A S E Y M D L

777 I S V L C E G S Y E S O R W P S L A A A G V A G A C S S D L R A A S A E G S C A G T I A C O O R S V C D L
 689 R P - - - D S A Y - - - - - S T S K D Y A Y C S V V V I S D E C E C I I A T E V X D L
 690 R P - - - D S V Y - - - - - S T S K D T K Y O S V V V I S E E K D E C C I I A T E V X D L
 691 R P - - - E S V Y - - - - - S T S K D T L Y C S V V V I S E E F K O F C V I A T E V X D L
 727 V C G T P W R
 728
 729
 730
 731

Met Gly Ser Arg Cys Ala Leu Ala Leu Ala Val Leu Ser Ala Leu Leu
 1 5 10 15
 Cys Gln Val Trp Ser Ser Gly Val Phe Glu Leu Lys Leu Gln Glu Phe
 20 25 30
 Val Asn Lys Lys Gly Leu Leu Gly Asn Arg Asn Cys Cys Arg Gly Gly
 35 40 45
 Ala Gly Pro Pro Pro Cys Ala Cys Arg Thr Phe Phe Arg Val Cys Leu
 50 55 60
 Lys His Tyr Gln Ala Ser Val Ser Pro Glu Pro Pro Cys Thr Tyr Gly
 65 70 75 80
 Ser Ala Val Thr Pro Val Leu Gly Val Asp Ser Phe Ser Leu Pro Asp
 85 90 95
 Gly Gly Gly Ala Asp Ser Ala Phe Ser Asn Pro Ile Arg Phe Pro Phe
 100 105 110
 Gly Phe Thr Trp Pro Gly Thr Phe Ser Leu Ile Ile Glu Ala Leu His
 115 120 125
 Thr Asp Ser Pro Asp Asp Leu Ala Thr Glu Asn Pro Glu Arg Leu Ile
 130 135 140
 Ser Arg Leu Ala Thr Gln Arg His Leu Thr Val Gly Glu Glu Trp Ser
 145 150 155 160
 Gln Asp Leu His Ser Ser Gly Arg Thr Asp Leu Lys Tyr Ser Tyr Arg
 165 170 175
 Phe Val Cys Asp Glu His Tyr Tyr Gly Glu Gly Cys Ser Val Phe Cys
 180 185 190
 Arg Pro Arg Asp Asp Ala Phe Gly His Phe Thr Cys Gly Glu Arg Gly
 195 200 205
 Glu Lys Val Cys Asn Pro Gly Trp Lys Gly Pro Tyr Cys Thr Glu Pro
 210 215 220
 Ile Cys Leu Pro Gly Cys Asp Glu Gln His Gly Phe Cys Asp Lys Pro
 225 230 235 240
 Gly Glu Cys Lys Cys Arg Val Gly Trp Gln Gly Arg Tyr Cys Asp Glu
 245 250 255
 Cys Ile Arg Tyr Pro Gly Cys Leu His Gly Thr Cys Gln Gln Pro Trp
 260 265 270
 Gln Cys Asn Cys Gln Glu Gly Trp Gly Gly Leu Phe Cys Asn Gln Asp
 275 280 285
 Leu Asn Tyr Cys Thr His His Lys Pro Cys Lys Asn Gly Ala Thr Cys
 290 295 300
 Thr Asn Thr Gly Gln Gly Ser Tyr Thr Cys Ser Cys Arg Pro Gly Tyr
 305 310 315 320
 Thr Gly Ala Thr Cys Glu Leu Gly Ile Asp Glu Cys Asp Pro Ser Pro
 325 330 335
 Cys Lys Asn Gly Gly Ser Cys Thr Asp Leu Glu Asn Ser Tyr Ser Cys
 340 345 350
 Thr Cys Pro Pro Gly Phe Tyr Gly Lys Ile Cys Glu Leu Ser Ala Met
 355 360 365
 Thr Cys Ala Asp Gly Pro Cys Phe Asn Gly Gly Arg Cys Ser Asp Ser
 370 375 380
 Pro Asp Gly Gly Tyr Ser Cys Arg Cys Pro Val Gly Tyr Ser Gly Phe
 385 390 395 400
 Asn Cys Glu Lys Lys Ile Asp Tyr Cys Ser Ser Ser Pro Cys Ser Asn
 405 410 415
 Gly Ala Lys Cys Val Asp Leu Gly Asp Ala Tyr Leu Cys Arg Cys Gln
 420 425 430
 Ala Gly Phe Ser Gly Arg His Cys Asp Asp Asn Val Asp Asp Cys Ala
 435 440 445
 Ser Ser Pro Cys Ala Asn Gly Gly Thr Cys Arg Asp Gly Val Asn Asp

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450	455	460
Phe	Ser	Cys
Thr	Cys	Pro
Cys	Pro	Gly
Pro	Gly	Tyr
Gly	Thr	Gly
Arg	Asn	Cys
Asn	Cys	Ser
Ala		
465	470	475
480		
Pro	Val	Ser
Arg	Cys	Glu
Cys	His	Ala
His	Pro	Cys
Gly	Asn	His
Arg	Gly	Gly
Gly	Tyr	Val
His	Val	Cys
Glu	Gly	Glu
Arg	Tyr	Val
Gly	500	505
505		
510		
Gly	Pro	Asn
Pro	Cys	Gln
Gly	Phe	Leu
Asn	Leu	Leu
Cys	Pro	Glu
Gly	Leu	Leu
515	520	525
525		
530	535	540
540		
Val	Val	Asp
Asp	Leu	Thr
Leu	Glu	Lys
Glu	Leu	Gly
Lys	Gly	Gln
Gly	Gly	Gly
535	540	540
540		
Trp	Val	Ala
Ala	Val	Cys
Val	Ala	Gly
Cys	Gly	Val
Ala	Val	Ile
545	550	555
555		
560		
Gly	Cys	Ala
Ala	Ala	Val
Ala	Val	Val
Val	Val	Cys
Cys	Val	Arg
Arg	Leu	Arg
Gly	Leu	Leu
565	570	575
575		
Arg	Pro	Pro
Pro	Ala	Asp
Ala	Asp	Pro
Asp	Cys	Arg
Cys	Gly	Glu
Arg	Glu	Thr
Gly	Thr	Glu
580	585	590
590		
595	600	605
605		
Ala	Thr	Gln
Thr	Ile	Lys
Gln	Asn	Asn
Ile	Asn	Thr
Lys	Asn	Lys
Asn	Thr	Asn
Thr	Lys	Lys
Gly	Asp	Ala
Asp	Phe	Asp
Phe	His	Gly
His	Gly	Asp
Ser	Ala	Asp
Ala	Asp	Lys
Asp	Lys	Asn
Lys	Gly	Phe
Gly	Phe	Lys
Asn	Lys	Ala
610	615	620
620		
625	630	635
635		
640		
Tyr	Asn	Leu
Asn	Leu	Val
Leu	Gln	Asp
Gln	Asp	Leu
Asp	Lys	Gly
Lys	Asp	Asp
Asp	Thr	Thr
Thr	Ala	Val
Ala	Val	Arg
Arg	Asp	Asp
Asp	Thr	Ala
Thr	Ser	Val
Ser	Gly	Asp
Gly	Asp	Thr
Asp	Thr	Lys
Thr	Ser	Tyr
Ser	Asp	Asp
Asp	Thr	Lys
Thr	Lys	Tyr
Gln	Ser	Val
Ser	Tyr	Val
Tyr	Val	Ile
Val	Ile	Ser
Ile	Ser	Glu
Ser	Glu	Glu
Glu	Glu	Lys
Lys	Asp	Asp
Asp	Glu	Glu
Glu	Cys	Cys
Cys	Val	Val
Val	Ile	Ile
Ile	Ile	Ala
705	710	715
715		
720		
720		
Thr	Glu	Val

FIGURE 4A CON'T

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GAATTCCATT	TTAAGTTATA	CAAAACTGAT	TACCATAAAGT	GCGGTCGACT	GCTTTATTT	60
TTACGTTGTG	TGTGTTGGAA	AAATGCTAAA	ACATCAGTCT	ACAATTCTAT	ATATTGTTAT	120
TAAAGATTAA	TCCAACCAGC	AACCCAAGGA	CATATAAGCG	ATTTCCACTA	TTGCATCAGA	180
GCACTCGGCA	GGAAAGGCCT	AGCCACGGGG	AACATTAGAA	GCTACAGAAG	CATTGCAGAG	240
AAGAGAAGAT	CCCCCGCGCG	TCCGCCGCTG	TTCTAAGGAG	AGAAGTGGGG	GCCCCCCCAGG	300
CTCGCGCGTG	GAGCGAAGCA	GCATGGGCAG	TCGGTGCAGC	CTGGCCCTGG	CGGTGCTCTC	360
GGCCTTGCTG	TGTCAAGGTCT	GGAGCTCTGG	GGTGTTCGAA	CTGAAGCTGC	AGGAGTCGT	420
CAACAAGAAG	GGGCTGCTGG	GGAAACCGCAA	CTGCTGCCGC	GGGGGCGCGG	GGCCACCGCC	480
GTGCGCCTGC	CGGACCTTCT	TCCCGGTGTG	CCTCAAGCAC	TACCAGGCCA	GCGTGTCCCC	540
CGAGCCGCC	TGCACTACG	GCAGCGCCGT	CACCCCCGTG	CTGGCGTCTG	ACTCCTTCAG	600
TCTGCCGAC	GGGGGGGGCG	CCGACTCCGC	GTTCAGCAAC	CCCATCCGCT	TCCCCCTTCGG	660
CTTCACCTGG	CCGGGCACCT	TCTCTCTGAT	TATTGAAGCT	CTCCACACAG	ATTCTCTGA	720
TGACCTCGCA	ACAGAAAACC	CAGAAAGACT	CATCAGCCGC	CTGGCCACCC	AGAGGCACCT	780
GACGGTGGGC	GAGGAGTGGT	CCCAGGACCT	GCACAGCAGC	GGCCGCACGG	ACCTCAAGTA	840
CTCCTACCGC	TTCGTGTGTG	ACGAACACTA	CTACGGAGAG	GGCTGCTCCG	TTTCTGCCG	900
TCCCCGGGAC	GATGCCTTCG	GCCACTTCAC	CTGTGGGGAG	CGTGGGGAGA	AAGTGTGCAA	960
CCCTGGCTGG	AAAGGGCCCT	ACTGCACAGA	GCCGATCTGC	CTGCCTGGAT	GTGATGAGCA	1020
GCATGGATT	TGTGACAAAC	CAGGGGAATG	CAAGTGCAGA	GTGGGCTGGC	AGGGCCGGTA	1080
CTGTGACGAG	TGTATCCGCT	ATCCAGGCTG	TCTCCATGGC	ACCTGCCAGC	AGCCCTGGCA	1140
GTGCAACTGC	CAGGAAGGCT	GGGGGGGCGT	TTTCTGCAAC	CAGGACCTGA	ACTACTGCAC	1200
ACACCATAAG	CCCTGCAAGA	ATGGAGCCAC	CTGCACCAAC	ACGGGCCAGG	GGAGCTACAC	1260
TTGCTCTTGC	CGGCCTGGGT	ACACAGGTGC	CACCTGCGAG	CTGGGGATTG	ACGAGTGTGA	1320
CCCCAGCCCT	TGTAAGAACG	GAGGGAGCTG	CACGGATCTC	GAGAACAGCT	ACTCCTGTAC	1380
CTGCCCAACCC	GGCTTCTACG	GCAAAATCTG	TGAATTGAGT	GCCATGACCT	GTGCGGACGG	1440
CCCTTGCTTT	AACGGGGGTC	GGTGCTCAGA	CAGCCCCGAT	GGAGGGTACA	GCTGCCGCTG	1500
CCCCGTGGGC	TACTCCGGCT	TCAACTGTGA	GAAGAAAATT	GAECTACTGCA	GCTCTTCACC	1560
CTGTTCTAAT	GGTGCCAAGT	GTGTGGACCT	CGGTGATGCC	TACCTGTGCC	GCTGCCAGGC	1620
CGGCTTCTCG	GGGAGGCACT	GTGACGACAA	CGTGGACGAC	TGCGCCTCCT	CCCCGTGCGC	1680
CAACGGGGC	ACCTGCCGGG	ATGGCGTGA	CGACTTCTCC	TGCACCTGCC	CGCCTGGCTA	1740
CACGGGCAGG	AACTGCAGTG	CCCCCGTCAG	CAGGTGCGAG	CACGCACCCCT	GCCACAATGG	1800
GGCCACCTGC	CACGAGAGGG	GCCACGGCTA	TGTGTGCGAG	TGTGCCCGAG	GCTACGGGGG	1860
TCCCAACTGC	CAGTTCTGC	TCCCCGAGCT	GCCCCCGGGC	CCAGCGTGG	TGGACCTCAC	1920
TGAGAAGCTA	GAGGGCCAGG	GCGGGCCATT	CCCCTGGGTG	GCCGTGTGCC	CCGGGGTCAT	1980
CCTTGTCTC	ATGCTGCTGC	TGGGCTGTG	CGCTGTGGTG	GTCTGCGTCC	GGCTGAGGCT	2040
GCAGAACGAC	CGGCCCCCAG	CCGACCCCTG	CCGGGGGGAG	ACGGAGACCA	TGAACAAACCT	2100
GGCCAACCTGC	CAGCGTGAGA	AGGACATCTC	AGTCAGCCTC	ATCGGGGCCA	CGCAGATCAA	2160
GAACACCAAC	AAGAAGGCGG	ACTTCCACGG	GGACCACAGC	GGCGACAAGA	ATGGCTTCAA	2220
GGCCCGCTAC	CCAGCGGTGG	ACTATAACCT	CGTGCAGGAC	CTCAAGGGTG	ACGACACCGC	2280
CGTCAGGGAC	GCGCACAGCA	AGCGTGACAC	CAAGTGCCAG	CCCCAGGGCT	CCTCAGGGGA	2340
GGAGAAGGGG	ACCCCGACCA	CACTCAGGGG	TGGAGAAGCA	TCTGAAAGAA	AAAGGCCGGA	2400
CTCGGGCTGT	TCAACTTCAA	AAGACACCAA	GTACCAGTCG	GTGTACGTCA	TATCCGAGGA	2460
GAAGGATGAG	TGCGTCATAG	CAACTGAGGT	GTAAAATGGA	AGTGAGATGG	CAAGACTCCC	2520
GTTTCTCTTA	AAATAAGTAA	AATTCCAAGG	ATATATGCC	CAACGAATGC	TGCTGAAGAG	2580
GAGGGAGGCC	TCGTGGACTG	CTGCTGAGAA	ACCGAGTTCA	GACCGAGCAG	GTTCTCCTCC	2640
TGAGGTCTC	GACGCCTGCC	GACAGCCTGT	CGCGGCCCGG	CCGCCTGCCG	CACTGCCCTC	2700
CGTGACGTGG	CCGTTGCACT	ATGGACAGTT	GCTCTTAAGA	GAATATATAT	TTAAATGGGT	2760
GAACGTGATT	ACGCATAAGA	AGCATGCACT	GCCTGAGTGT	ATATTTGGA	TTCTTATGAG	2820
CCAGTCTTT	CTTGAATTAG	AAACACAAAC	ACTGCCTTA	TTGTCCCTTT	TGATACGAAG	2880
ATGTGCTTT	TCTAGATGGA	AAAGATGTGT	GTTATTTTT	GGATTTGTAA	AAATATTTT	2940
CATGATATCT	GTAAAGCTTG	AGTATTTGT	GATGTTCGTT	TTTTATAATT	TAAATTTGG	3000
TAAATATGTA	CAAAGGCAC	TCGGGTCTAT	GTGACTATAT	TTTTTTGTAT	ATAAAATGTAT	3060
TTATGGAATA	TTGTGCAAAT	GTTATTTGAG	TTTTTTACTG	TTTGTTAAT	GAAGAAATTC	3120
CTTTTAAAAA	TATTTTCCA	AAATAAATT	TATGAGGAAT	TC		3162

FIGURE 4B

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Gly Glu Glu Gly Ser Phe Ser His Gly Ser Val Ile Asp Gly Arg Phe
 1 5 10 15
 Glu Gly Phe Ile Gln Thr Arg Gly Gly Thr Phe Tyr Val Glu Pro Ala
 20 25 30
 Glu Arg Tyr Ile Lys Asp Arg Arg Thr Leu Pro Phe His Ser Val Ile Tyr
 35 40 45
 His Glu Asp Asp Ile Ser Glu Arg Leu Lys Leu Arg Leu Arg Lys Leu
 50 55 60
 Met Ser Leu Glu Leu Trp Thr Ser Cys Cys Leu Pro Cys Ala Leu Leu
 65 70 75 80
 Leu His Ser Trp Lys Lys Ala Val Asn Ser His Cys Leu Tyr Phe Lys
 85 90 95
 Asp Phe Trp Gly Phe Ser Glu Ile Tyr Tyr Pro His Lys Tyr Gly Pro
 100 105 110
 Gln Gly Gly Cys Ala Asp His Ser Val Phe Glu Arg Met Arg Lys Tyr
 115 120 125
 Gln Met Thr Gly Val Glu Glu Val Thr Gln Ile Pro Gln Glu Glu His
 130 135 140
 Ala Ala Asn Gly Pro Glu Leu Leu Arg Lys Arg Arg Thr Thr Ser Ala
 145 150 155 160
 Glu Lys Asn Thr Cys Gln Leu Tyr Ile Gln Thr Asp His Leu Phe Phe
 165 170 175
 Lys Tyr Tyr Gly Thr Arg Glu Ala Val Ile Ala Gln Ile Ser Ser His
 180 185 190
 Val Lys Ala Ile Asp Thr Ile Tyr Gln Thr Thr Asp Phe Ser Gly Ile
 195 200 205
 Arg Asn Ile Ser Phe Met Val Lys Arg Ile Arg Ile Asn Thr Thr Ala
 210 215 220
 Asp Glu Lys Asp Pro Thr Asn Pro Phe Arg Phe Pro Asn Ile Ser Val
 225 230 235 240
 Glu Lys Phe Leu Glu Leu Asn Ser Glu Gln Asn His Asp Asp Tyr Cys
 245 250 255
 Leu Ala Tyr Val Phe Thr Asp Arg Asp Phe Asp Asp Gly Val Leu Gly
 260 265 270
 Leu Ala Trp Val Gly Ala Pro Ser Gly Ser Ser Gly Gly Ile Cys Glu
 275 280 285
 Lys Ser Lys Leu Tyr Ser Asp Gly Lys Lys Lys Ser Leu Asn Thr Gly
 290 295 300
 Ile Ile Thr Val Gln Asn Tyr Gly Ser His Val Pro Pro Lys Val Ser
 305 310 315 320
 His Ile Thr Phe Ala His Glu Val Gly His Asn Phe Gly Ser Pro His
 325 330 335
 Asp Ser Gly Thr Glu Cys Thr Pro Gly Glu Ser Lys Asn Leu Gly Gln
 340 345 350
 Lys Glu Asn Gly Asn Tyr Ile Met Tyr Ala Arg Ala Thr Ser Gly Asp
 355 360 365
 Lys Leu Asn Asn Asn Lys Phe Ser Leu Cys Ser Ile Arg Asn Ile Ser
 370 375 380
 Gln Val Leu Glu Lys Lys Arg Asn Asn Cys Phe Val Glu Ser Gly Gln
 385 390 395 400
 Pro Ile Cys Gly Asn Gly Met Val Glu Gln Gly Glu Glu Cys Asp Cys
 405 410 415
 Gly Tyr Ser Asp Gln Cys Lys Asp Glu Cys Cys Phe Asp Ala Asn Gln
 420 425 430
 Pro Glu Gly Arg Lys Cys Lys Leu Lys Pro Gly Lys Gln Cys Ser Pro
 435 440 445
 Ser Gln Gly Pro Cys Cys Thr Ala Gln Cys Ala Phe Lys Ser Lys Ser
 450 455 460

FIGURE 5A

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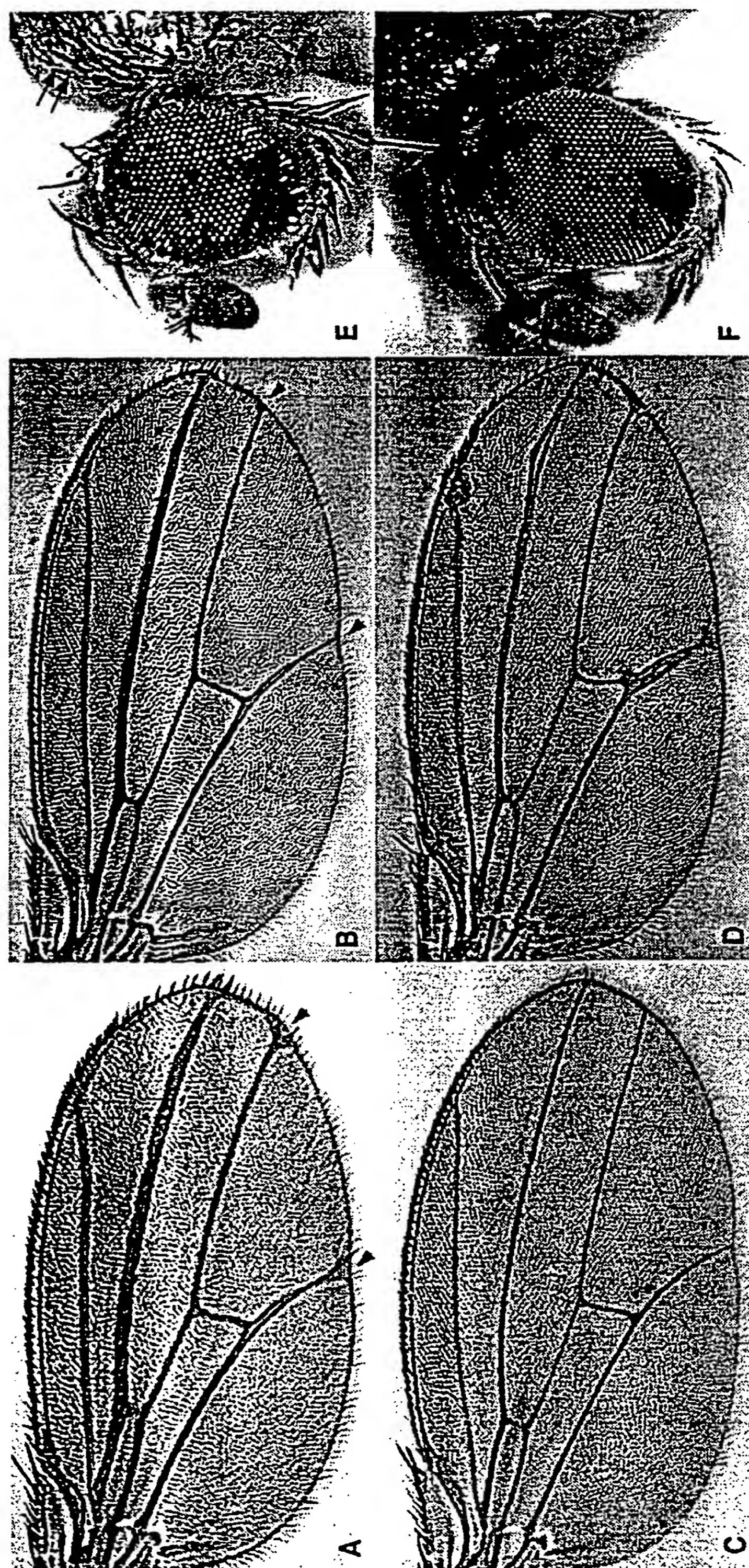
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465 470 475 480
Gly Phe Thr Ala Leu Cys Pro Ala Ser Asp Pro Lys Pro Asn Phe Thr
485 490 495
Asp Cys Asn Arg His Thr Gln Val Cys Ile Asn Gly Gln Cys Ala Gly
500 505 510
Ser Ile Cys Glu Lys Tyr Gly Leu Glu Cys Thr Cys Ala Ser Ser
515 520 525
Asp Gly Lys Asp Asp Lys Glu Leu Cys His Val Cys Cys Met Lys Lys
530 535 540
Met Asp Pro Ser Thr Cys Ala Ser Thr Gly Ser Val Gln Trp Ser Arg
545 550 555 560
His Phe Ser Gly Arg Thr Ile Thr Leu Gln Pro Gly Ser Pro Cys Asn
565 570 575
Asp Phe Arg Gly Tyr Cys Asp Val Phe Met Arg Cys Arg Leu Val Asp
580 585 590
Ala Asp Gly Pro Leu Ala Arg Leu Lys Lys Ala Ile Phe Ser Pro Glu
595 600 605
Leu Tyr Glu Asn Ile Ala Glu Trp Ile Val Ala His Trp Trp Ala Val
610 615 620
Leu Leu Met Gly Ile Ala Leu Ile Met Leu Met Ala Gly Phe Ile Lys
625 630 635 640
Ile Cys Ser Val His Thr Pro Ser Ser Asn Pro Lys Leu Pro Pro Pro
645 650 655
Lys Pro Leu Pro Gly Thr Leu Lys Arg Arg Arg Pro Pro Gln Pro Ile
660 665 670
Gln Gln Pro Gln Arg Gln Arg Pro Arg Glu Ser Tyr Gln Met Gly His
675 680 685
Met Arg Arg
690

FIGURE 5A CON'T

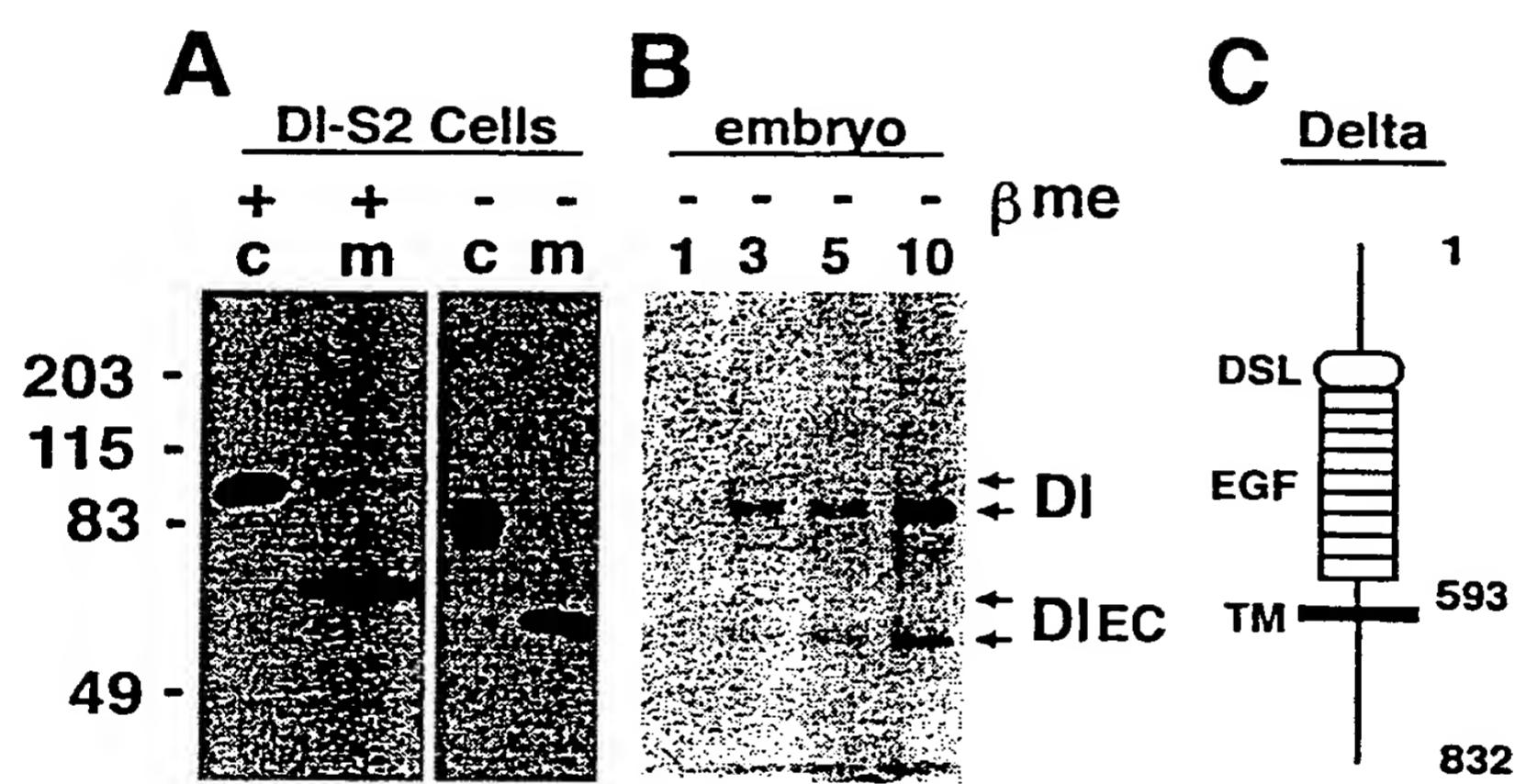
10/16

GGTGAAGAAG	GAAGTTTAG	CCATGGGTCT	GTTATTGATG	GAAGATTGA	AGGATTCA	60
CAGACTCGTG	GTGGCACATT	TTATGTTGAG	CCAGCAGAGA	GATATATTAA	AGACCGAACT	120
CTGCCATTTC	ACTCTGTCAT	TTATCATGAA	GATGATATTA	GTGAAAGGCT	TAAACTGAGG	180
CTTAGAAAAC	TTATGTCACT	TGAGTTGTGG	ACCTCCTGTT	GTTCACCTG	TGCTCTTCTG	240
CTTCACTCAT	GGAAGAAAGC	TGTAATTC	CACTGCCTT	ACTTCAAGGA	TTTCTGGGC	300
TTTTCTGAAA	TCTACTATCC	CCATAAATAC	GGTCCTCAGG	GCGGCTGTGC	AGATCATTCA	360
GTATTTGAAA	GAATGAGGAA	ATACCAGATG	ACTGGGTGAG	AGGAAGTAAC	ACAGATACCT	420
CAAGAAGAAC	ATGCTGCTAA	TGGTCCAGAA	CTTCTGAGGA	AAAGACGTAC	AACTTCAGCT	480
GAAAAAAAATA	CTTGTCAAGCT	TTATATTCA	ACTGATCATT	TGTTCTTAA	ATATTACGGA	540
ACACGAGAAC	CTGTGATTGC	CCAGATATCC	AGTCATGTTA	AAGCGATTGA	TACAATTAC	600
CAGACCACAG	ACTTCTCCGG	AATCCGTAAC	ATCAGTTCA	TGGTGAACG	CATAAGAAC	660
AATACAAC	CTGATGAGAA	GGACCCTACA	AATCCTTCC	GTTCACCTAA	TATTAGTGTG	720
GAGAAGTTTC	TGGAATTGAA	TTCTGAGCAG	AATCATGATG	ACTACTGTT	GGCCTATGTC	780
TTCACAGACC	GAGATTTGA	TGATGGCGTA	CTTGGTCTGG	CTTGGGTTGG	AGCACCTCA	840
GGAAGCTCTG	GAGGAATATG	TGAAAAAAAGT	AAACTCTATT	CAGATGGTAA	GAAGAAGTCC	900
TTAAACACTG	GAATTATTAC	TGTCAGAAC	TATGGGTCTC	ATGTACCTCC	CAAAGTCTCT	960
CACATTACTT	TTGCTCACGA	AGTTGGACAT	AACTTTGGAT	CCCCACATGA	TTCTGGAACA	1020
GAGTGCACAC	CAGGAGAAC	TAAGAATTG	GGTCAAAAG	AAAATGGCAA	TTACATCATG	1080
TATGCAAGAG	CAACATCTGG	GGACAAAATT	AACAACAATA	AATTCTCA	CTGTAGTATT	1140
AGAAAATATAA	GCCAAGTTCT	TGAGAAGAAG	AGAAACAACT	GTTTGTTGA	ATCTGGCCAA	1200
CCTATTGTTG	GAAATGGAAT	GGTAGAACAA	GGTGAAGAAT	GTGATTGTGG	CTATAGTGA	1260
CAGTGTAAAG	ATGAATGCTG	CTTCGATGCA	AATCAACCAG	AGGGAAAGAAA	ATGCAAAC	1320
AAACCTGGGA	AACAGTGCAG	TCCAAGTC	GGTCCTTGT	GTACAGCACA	GTGTGCATTC	1380
AAGTCAAAGT	CTGAGAAGTG	TCGGGATGAT	TCAGACTGTG	CAAGGGAAAGG	AATATGTAAT	1440
GGCTTCACAG	CTCTCTGCC	AGCATCTGAC	CCTAAACCAA	ACTTCACAGA	CTGTAATAGG	1500
CATACACAAG	TGTGCATTAA	TGGGCAATGT	GCAGGTTCTA	TCTGTGAGAA	ATATGGCTTA	1560
GAGGAGTGT	CGTGTGCCAG	TTCTGATGGC	AAAGATGATA	AAGAATTATG	CCATGTATGC	1620
TGTATGAAGA	AAATGGACCC	ATCAACTTGT	GCCAGTACAG	GGTCTGTGCA	GTGGAGTAGG	1680
CACTTCAGTG	GTCGAACCAT	CACCCTGCAA	CCTGGATCCC	CTTGCAACGA	TTTAGAGGT	1740
TACTGTGATG	TTTCATGCG	GTGCAGATTA	GTAGATGCTG	ATGGTCCTCT	AGCTAGGCTT	1800
AAAAAAAGCAA	TTTTAGTCC	AGAGCTCTAT	AAAAACATTG	CTGAATGGAT	TGTGGCTCAT	1860
TGGTGGGCAG	TATTACTTAT	GGGAATTGCT	CTGATCATGC	TAATGGCTGG	ATTATTAAG	1920
ATATGCAGTG	TTCATACTCC	AAAGTAGTAAT	CCAAAGTTGC	CTCCTCCTAA	ACCACTTCCA	1980
GGCACTTTAA	AGAGGAGGAG	ACCTCCACAG	CCCATTCA	AACCCCA	TCAGGGCCC	2040
CGAGAGAGTT	ATCAAATGGG	ACACATGAGA	CGCTAATGC	AGCTTTGCC	TTGGTTCTTC	2100
CTAGTGCCTA	CAATGGGAAA	ACTTCACTCC	AAAGAGAAAC	CTATTAAGTC	ATCATCTCCA	2160
AACTAAACCC	TCACAAGTAA	CAGTTGAAGA	AAAAATGGCA	AGAGATCATA	TCCTCAGACC	2220
AGGTGGAATT	ACTTAAATT	TAAAGCCTGA	AAATTCCAAT	TTGGGGGTGG	GAGGTGGAAA	2280
AGGAACCCAA	TTTCTTATG	AACAGATATT	TTAACCTAA	TGGCACAAAG	TCTTAGAATA	2340
TTATTATGTG	CCCCGTGTT	CCTGTTCTC	GTTGCTGCAT	TTTCTTC	TGCAGGCAA	2400
CTTGGCTCTC	AATAAAC	TCG				2423

FIGURE 5B

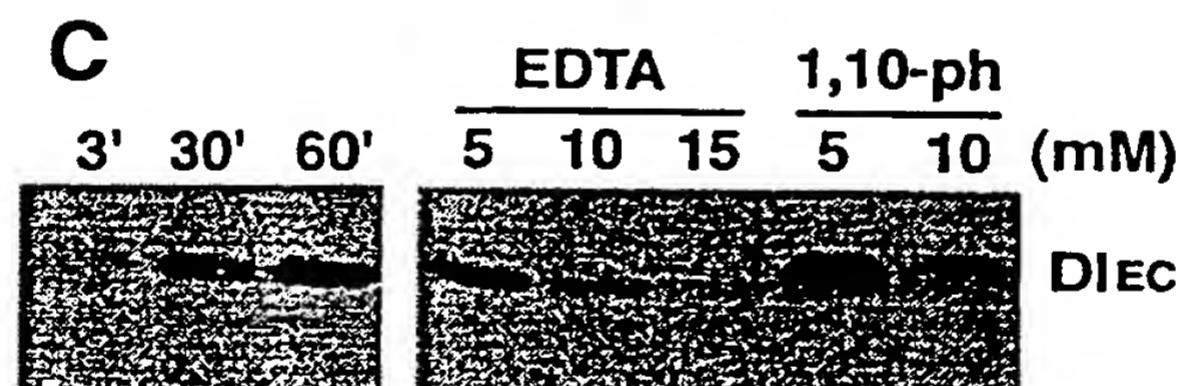
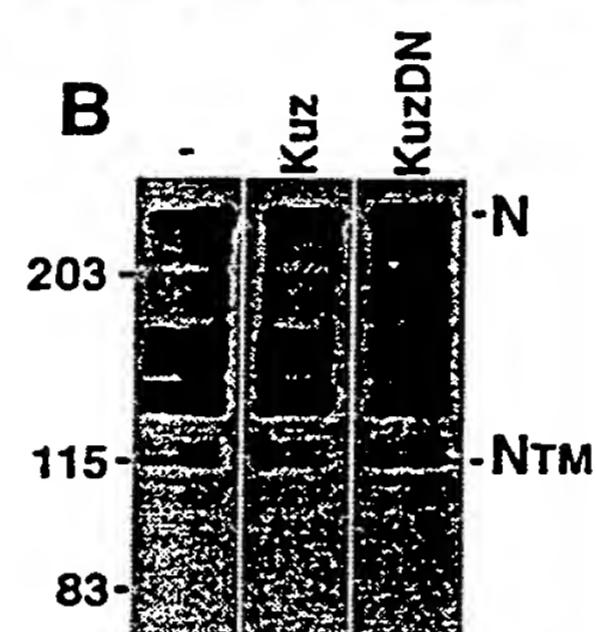
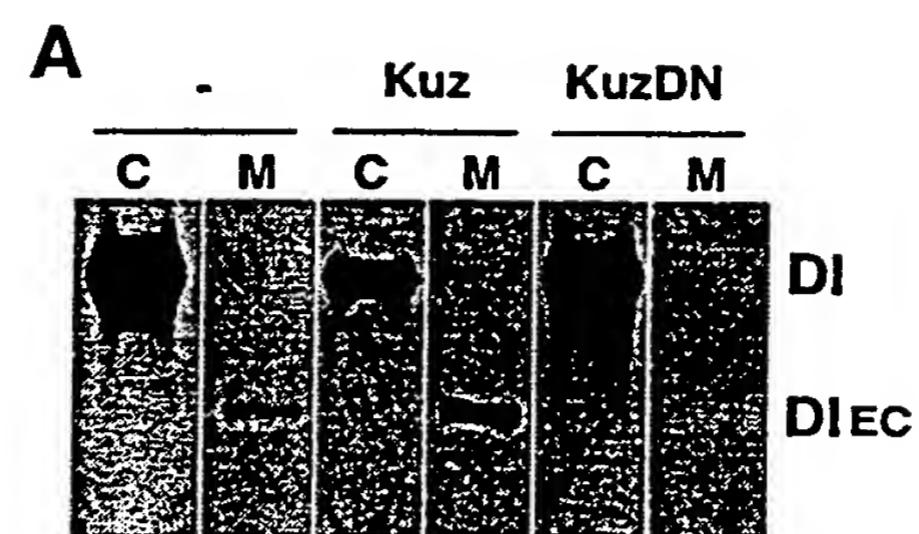


FIGURES 6A - 6F

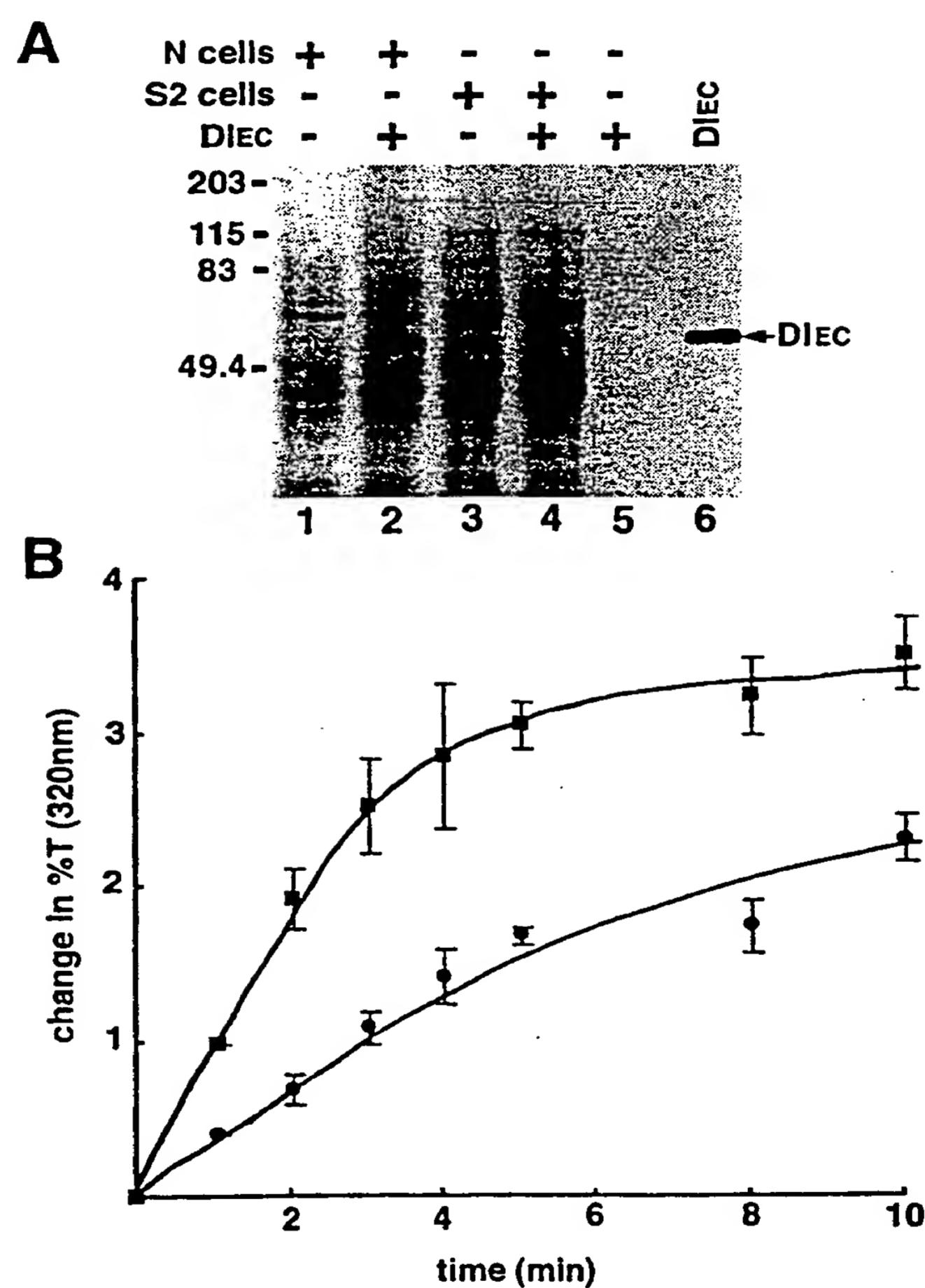


FIGURES 7A-7C

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FIGURES 8A - 8D



FIGURES 9A - 9B

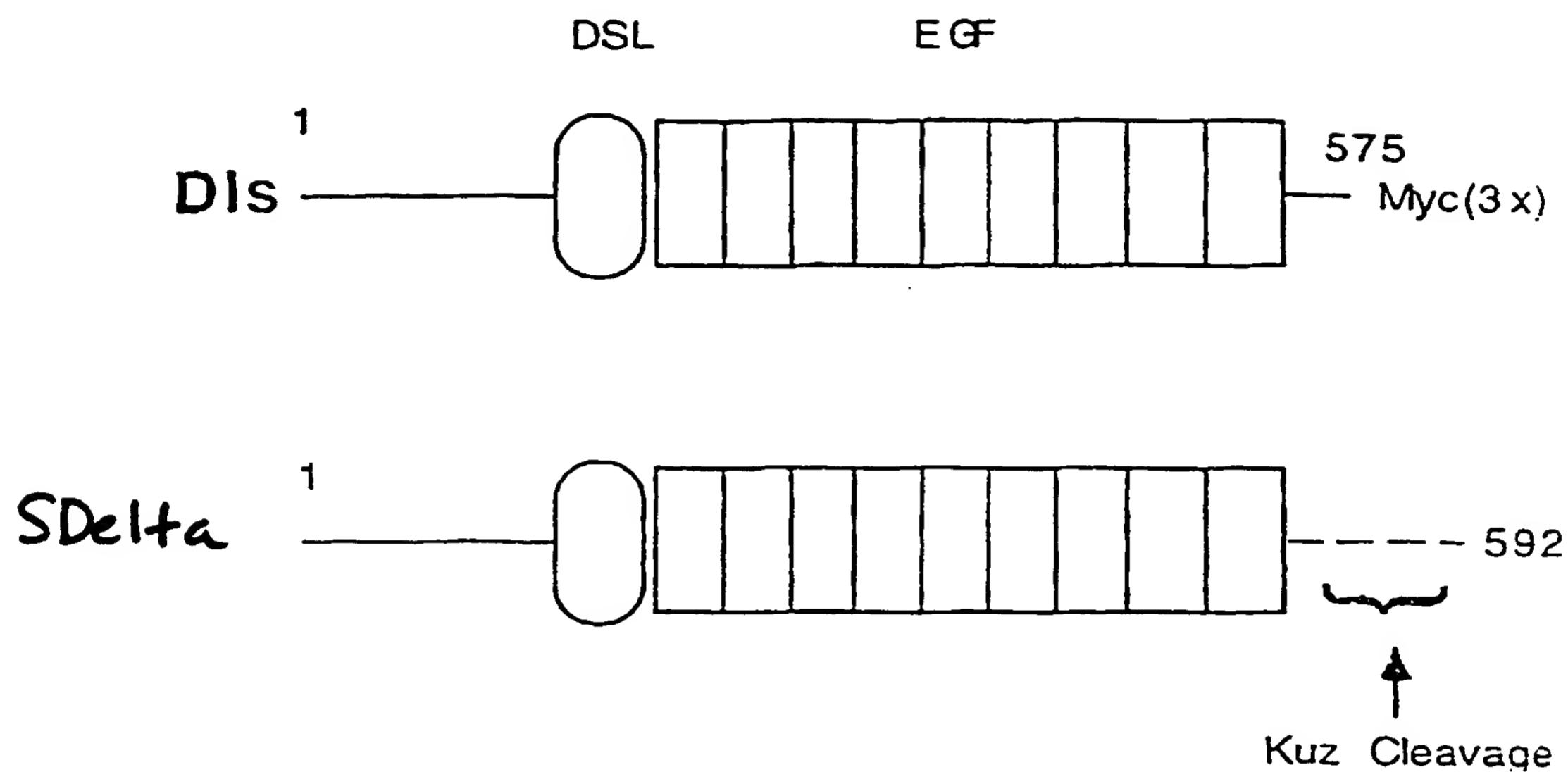


FIGURE 10

C₅₆₄DEESYDSVTFDAHQYGATTQARADGLTNAQ₅₉₄
(A)

FIGURE 11